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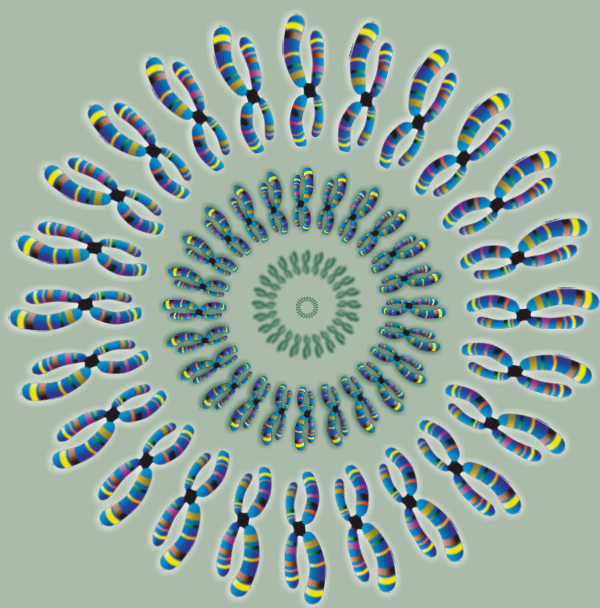

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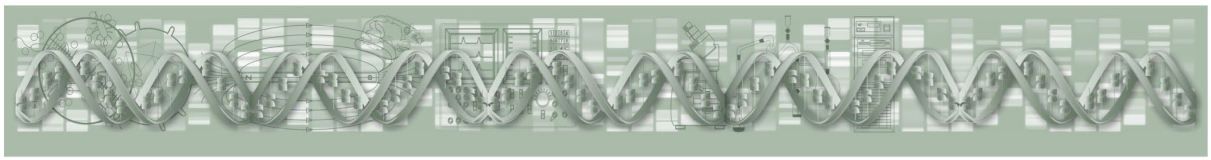

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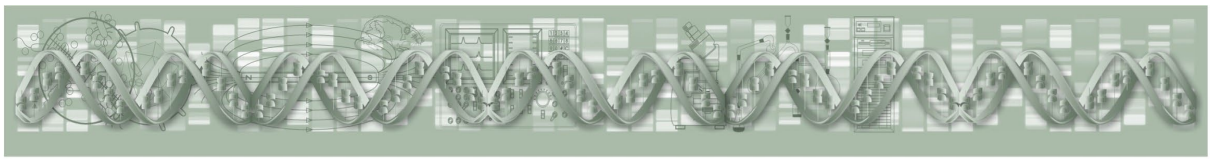

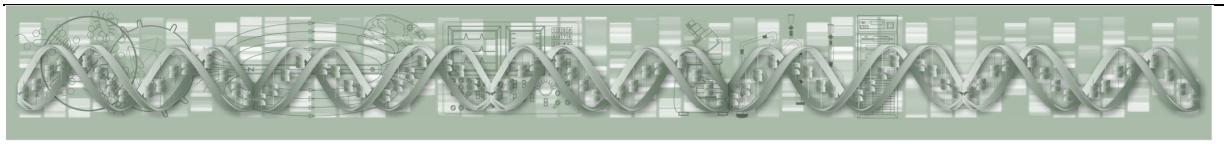

ASSESSMENT OF THE EFFECTS OF METHANOL LEAF EXTRACT OF *CLERODENDRUM VIOLACEUM* ON THE LIVER OF MICE

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Abstract

Effects of methanol leaf extract of *Clerodendrum violaceum* on liver function indices of Swiss mice was evaluated. Six groups (A-F) of ten mice each were used. Groups B-F were administered 31.25, 62.5, 125, 250, 500 mg/kg body weight of methanol leaf extract of *Clerodendrum violaceum*, respectively. Group A received 5% DMSO (control). Five animals in each group were sacrificed after 14 days of administration; the remaining were sacrificed after 28 days of administration. Blood was collected for analyses, livers were collected and weighed. Some of the liver samples were homogenized and some preserved in 10% formalin for histopathological examination. After 14 days, there was significant increase ($p < 0.05$) in total and conjugated bilirubin and significant reduction ($p < 0.05$) of albumin and total protein at higher doses. Activities of ALP and γ-GT in serum were significantly elevated ($p < 0.05$) at all doses while liver and serum ALT activity only at lower doses. Liver and serum AST activity were also significantly elevated ($p < 0.05$) at higher doses. Activities of ALP and γ-GT in the liver were significantly reduced ($p < 0.05$) at all doses while ALT activity only reduced at the highest dose in liver and serum. AST activity was reduced at higher doses in liver but only at highest dose in serum. Liver tissue was inflamed with progressive degeneration on day 28. Results showed that methanol leaf extract of *C. violaceum* adversely affected the normal architecture, synthetic and secretory functions of the liver at high doses.

Keywords: *Clerodendrum violaceum,* histopathological screening, liver function indices, organ-body weight ratio

Introduction

The liver is a large organ made up of chemically reactant pool of cells having a high rate of metabolism. It is responsible for sharing substrates and energy between metabolic systems, processing and synthesizing multiple important substances for transport to other areas of the body and is involved in several other metabolic functions (Dutta et al. 2021). The liver plays a major role in carbohydrate, amino acid and lipid metabolism and plays a key role in the biotransformation of foods, toxic substances, and medicinal products (Arman et al. 2022). Due to these diverse and essential functions carried out by the liver, any change in its normal structure or function will have far reaching consequences. Some of these changes have been shown to occur during disease or exposure to drugs, chemicals, and toxicants, including medicinal plants (Intagliata and Caldwell 2017, Nunes et al. 2022). Monitoring of potential adverse effects of drugs and other compounds on the liver is therefore vital in diagnosis, recovery, and follow-up of many medical conditions (Lilford et al. 2013, Liao et al. 2022).
Medicinal plants have been identified and used to treat various ailments even before the advent of orthodox medicines (Sofowora et al. 2013). Plants produce several chemical compounds, (secondary metabolites) some of which have been shown to have pharmacological effects.

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However, the presence of these compounds in most medicinal plants may lead to complex and sometimes detrimental effects (Obeten et al. 2017, Okaiyeto and Oguntibeju 2021). The liver is uniquely situated to take up and process all chemical compounds coming into the blood since the metabolism of drugs and other exogenous compounds, including medicinal preparations, mainly takes place there. It is thus, liable to adverse effects from such drugs and their metabolites (García and García 2022).

Clerodendrum violaceum (*C. violaceum*) (Verbenaceae) is commonly called Clerodendrum in English and `Ewe isedun´ in Yoruba (Nigeria). A decoction of its leaves is used for the treatment of fever/malaria in folk medicine. We have previously authenticated the acclaimed antimalarial activity of its leaf extract (Adebayo et al. 2022). We have also reported that its antimalarial activity is augmented by its antioxidant activity (Balogun et al. 2014).

Since *C. violaceum* is taken traditionally for fever and has been shown to have antimalarial activity and considering the physiological roles of the liver in health and disease, it is of interest to investigate its effect on the liver and its function indices.

Materials and Methods

Methanol was obtained from BDH Laboratory Supplies, Poole Dorset BH15 UK. Assay kits for enzymes were obtained from Randox Laboratories Ltd. (Co. Antrim, U.K). All other reagents used were of analar grade and prepared in all glass distilled water.

Plant materials

Fresh leaf samples of *C. violaceum* were collected in Oyo town, Oyo State, Nigeria and were botanically authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria. A specimen with voucher number FHI 108879 was deposited.

Animals

Sixty (60) adult Swiss laboratory mice with an average weight of 20 ± 2 g were obtained from the Animal Breeding Unit of the Department of Biochemistry, University of Jos, Plateau State, Nigeria. The mice were housed in plastic cages and maintained under standard laboratory conditions with free access to rat pellets and tap water *ad libitum*. Animal care and experiments/procedures were carried out according to the ethical guidelines of the NNREC (Norwegian National Research Ethics Committee (2019).

Plant extracts preparation

Fresh leaves of the plant were dried in the shade for seven days at room temperature (25±2 °C) and pulverized to powder using an electric blender (Mazeda Mill, MT 4100, Japan). Four hundred and fifty grams (450 g) of the powder was exhaustively extracted with 4 L n-hexane, 4 L ethyl acetate and 4 L absolute methanol successively for 72 h each. The extracts were filtered using Whatman filter paper No 1 and concentrated under pressure after each extraction period using a rotary evaporator. The concentrates were then exposed to air and allowed to evaporate at room temperature to dryness (Adebayo et al. 2003). In our previous study, methanol extract had the highest antioxidant activity (Balogun et al. 2014) and the best antimalarial efficacy (Adebayo et al. 2022); hence, only the methanol extract was used in this study.

Experimental Design

Sixty Swiss laboratory mice were randomly divided into six groups (A-F) of ten mice each and given the methanol leaf extract of *C. violaceum* orally as follows:

Animals in group A received 5% DMSO and served as control; those in groups B, C, D, E and F received 31.25, 62.5, 125, 250 and 500 mg/kg body weight of the methanol leaf extract of *C. violaceum*, respectively. After fourteen days of extract administration, five animals from each group were sacrificed; blood and liver tissue were collected for analysis. Extract administration

continued for another fourteen days after which the remaining animals in all the groups were sacrificed and treated similar to the first batch.

Collection of Blood Samples, Liver Tissue and preparation of Serum and Tissue Supernatants

The mice were sacrificed following diethyl ether anaesthesia. Blood was collected by cardiac puncture into clean, dry test tubes and was allowed to stand for about fifteen minutes at room temperature to clot. It was then centrifuged at 1000 rpm (Gallenkamp Centrifuge 200) for fifteen minutes. The clear supernatant (serum) was carefully collected with a Pasteur pipette. The animals were then dissected, and the liver was removed, cleaned and weighed. The liver samples for each group were then homogenized separately in ice-cold 0.25 M sucrose solution (1:5 w/v). The homogenates were stored frozen overnight before centrifuging. The supernatant obtained after centrifuging was used in the analyses. Parts of the weighed liver tissues were also collected in specimen bottles containing 10% formalin and fixed for histopathological examination.

Organ-body weight ratio

After the mice were sacrificed, their livers were immediately removed, cleaned, and weighed. The relative liver weights were calculated by dividing the weights of livers by the final body weights of the corresponding animals before sacrifice.

Histopathological studies

The method of Krause (2001) which includes several processing steps was used to assess the histopathological effect(s) of the methanol leaf extract of *C. violaceum* on the liver of mice. Briefly, after sacrificing the mice, liver tissue samples were collected and fixed in 10 % formalin to preserve tissues and maintain lifelike structures. The samples were then transported to the Histology laboratory of the Department of Anatomy, College of Health Sciences, University of Ilorin, Kwara State, Nigeria where the processing, preparation, and interpretation of histopathology slides was carried out. The fixed tissue samples were first dehydrated to remove excess water and formalin by immersing them in ascending grades of alcohol. They were then cleared with xylene to remove the alcohol and impregnated with molten paraffin which infiltrates the tissue samples, replaces the clearing agent and provides support. Next, the tissue samples were embedded with more paraffin. Embedding enables careful positioning of the tissue inside a base mould. The tissues were then sectioned from the paraffin block with a rotary microtome and the cut sections were placed on a microscope slide, dried and stained with Hematoxylin and Eosin (H/E) to highlight important features of the tissues and enhance contrast. A Synthetic mountant was added to a coverslip and placed on top of the tissue section on the slide to keep the specimen in place and protect from any accidental contact. Images of the sections were then captured using a camera attached to a microscope (Omax-MD82ES10). Photomicrographs were captured at x400 magnification.

Analysis of biochemical parameters

The method of Ueno et al. (2013) was used to determine albumin concentration. Protein concentrations were determined using the method reported by Zheng et al. (2017). For serum bilirubin, the method reported by Kalakonda et al. 2022 was used. Alkaline phosphatase activity was determined as described by Wright et al. (1972) while the method of Corti et al. (2019) was used to assay for gamma glutamyl transferase activity. The activities of alanine and aspartate aminotransferases were assayed by the method described by Reitman and Frankel (1957).

Statistical analysis

The group means ± Standard Deviation (SD) for each parameter was calculated and significant differences were determined by Analysis of Variance (ANOVA). Duncan's Multiple Range

Test (DMRT) was used for post-hoc test at 95% confidence level using SPSS-PC programme packages (Version 24.0, SPSS Inc. Chicago).

Results and discussion

Liver Function indices

Administration of the extract for 14 days caused a significant reduction ($p < 0.05$) in serum albumin at 500 mg/kg body weight while serum total protein concentration was significantly decreased ($p < 0.05$) at the doses of 250 and 500 mg/kg body weight compared to controls. After 28 days of extract administration, there was an increase in the concentration of albumin at the dose of 250 mg/kg body weight while it was reduced significantly ($p < 0.05$) at 500 mg/kg body weight compared to control. However, serum total protein concentration reduced significantly ($p < 0.05$) at all doses compared to control. There was significant increase ($p < 0.05$) in total bilirubin concentration at the dose of 250 and 500 mg/kg body weight while conjugated bilirubin concentration was increased significantly ($p < 0.05$) at all doses compared to controls after 14 days of extract administration. After 28 days, there was a significant ($p < 0.05$) increase in total bilirubin concentration while conjugated bilirubin concentration was not significantly altered ($p > 0.05$) compared to controls at all doses of the extract administered (Tables 1 and 2).

Table 1. Effects of Methanol Leaf Extract of *Clerodendrum violaceum* on Liver Function Indices of Mice after 14 Days of Administration

Treatment	Albumin (g/dL)	Total protein (mg/ml)	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)
Control	13.23± 0.57^{a}	60.18± 1.04^{a}	1.38 ± 0.11^{a}	0.38 ± 0.10^{a}
31.25 mg/kg b. wt	12.45± 0.39^{a}	59.28± 2.65^{a}	1.42 ± 0.15^{a}	0.51 ± 0.24^{b}
62.5 mg/kg b. wt	11.38± 0.58^{a}	56.85± 2.02^{a}	1.44 ± 0.29^{a}	0.53 ± 0.11^{b}
125 mg/kg b. wt	10.30± 0.52^{a}	52.45± 1.14^{a}	1.50 ± 0.13^{a}	0.59 ± 0.17^{b}
250 mg/kg b. wt	10.00± 0.10^{a}	48.58±1.81^{b}	1.55 ± 0.17^{b}	0.60 ± 0.31^{b}
500 mg/kg b. wt	6.00 ± 0.23^{b}	32.25± 1.26^{c}	1.76 ± 0.13^{b}	0.80 ± 0.19^{c}

Values are means of 5 replicates ±SD. Means in the same column with different superscripts for each parameter are significantly different ($p < 0.05$).

Table 2. Effects of Methanol Leaf Extract of *C. violaceum* on Liver Function Indices of Mice after 28 days of Administration

Treatment	Albumin (g/dL)	Total protein (mg/ml)	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)
Control	15.28±0.34^{a}	67.90±1.38^{a}	1.39±0.06^{a}	0.49±0.07^{a}
31.25 mg/kg b. wt	17.28±0.65^{a}	54.95±2.63^{b}	1.46±0.10^{a}	0.53±0.03^{a}
62.5 mg/kg b. wt	13.65±0.56^{a}	53.85±1.86^{b}	1.48±0.17^{a}	0.55±0.05^{a}

125 mg/kg b. wt	15.25±0.39[a]	50.50±1.12[b]	1.54±0.12[b]	0.60±0.06[a]
250 mg/kg b. wt	20.35±0.81[b]	47.45±1.15[b]	1.57±0.13[b]	0.73±0.03[a]
500 mg/kg b. wt	8.00±0.24[c]	25.92±1.56[c]	1.70±0.12[c]	0.68±0.04[a]

Results are means of 5 determinations ±SD. Means along the same column with different superscripts for each parameter are significantly different ($p < 0.05$).

The concentrations of albumin, total protein and bilirubin in the blood can be used to gain information on the the state of the liver (Sawieres 2022).

Albumin along with other plasma proteins cannot normally diffuse through the thin capillary wall membranes since they are colloidal molecules. Therefore, they remain trapped in the vascular system where they exert a colloidal osmotic pressure which helps to maintain a normal blood volume (Moman et al. 2022). Thus, a decrease in serum albumin concentration which was significant at 500 mg/kg body weight throughout the days of extract administration might be due to a diminished synthetic function of the liver. Any Liver injury can lead to a disturbance in its physiological roles which includes the ability to synthesize albumin at a rate commensurate with catabolism resulting in a reduction in albumin concentration (Ugwu and Suru 2021). Since albumin plays an important role in maintaining an oncotic pressure difference between the plasma and interstitial space, any decrease in serum albumin concentration if left unchecked will cause the diffusion of water from the blood vessels into the interstitial fluid and tissues (Adebayo et al. 2009, Shi et al. 2022). The significant decrease in the total protein concentration at 250 and 500 mg/kg body weight on day 14 (Table 1) and at all doses after 28 days of extract administration (Table 2) may be for the same reason.

Bilirubin is the main bile pigment formed from the breakdown of haem in red blood cells. The serum bilirubin concentration is considered a true test of liver function because it reflects the ability of the liver to take up, process and secrete bilirubin. The significant increase in serum total bilirubin concentration at 250 and 500 mg/kg body weight and the significant increase at all doses for serum conjugated bilirubin concentration after 14 days of extract administration (Table 1) could be an indication of an impairment in the functional capacity of the liver and possibly haemolysis especially at higher doses (Nunes et al. 2022). Jaundice is caused either due to overproduction of bilirubin or inability of the liver to clear it and is found in several diseases including heamolytic anaemia, cholestasis, Gilbert's syndrome, malaria, and inflammation (Janghel et al. 2019). The increase in serum total bilirubin suggests that higher doses of the extract should be used with caution as it may lead to hyperbilirubinaemia.

Significant elevation of total bilirubin concentration at all doses on day 28 (Table 2) could be a cumulative effect of the extract on the liver leading to an accumulation of bilirubin since the conjugated bilirubin was not affected. These reductions suggest that using this extract at higher doses or for prolonged periods may affect liver function.

Cellular Enzymes

Alkaline Phosphatase

There was a dose-dependent significant increase ($p < 0.05$) in ALP activity in the serum compared to controls after 14 days of extract administration (Figure 1). There was also dose-dependent significant decrease ($p < 0.05$) in liver ALP activity compared to control (Figure 1).

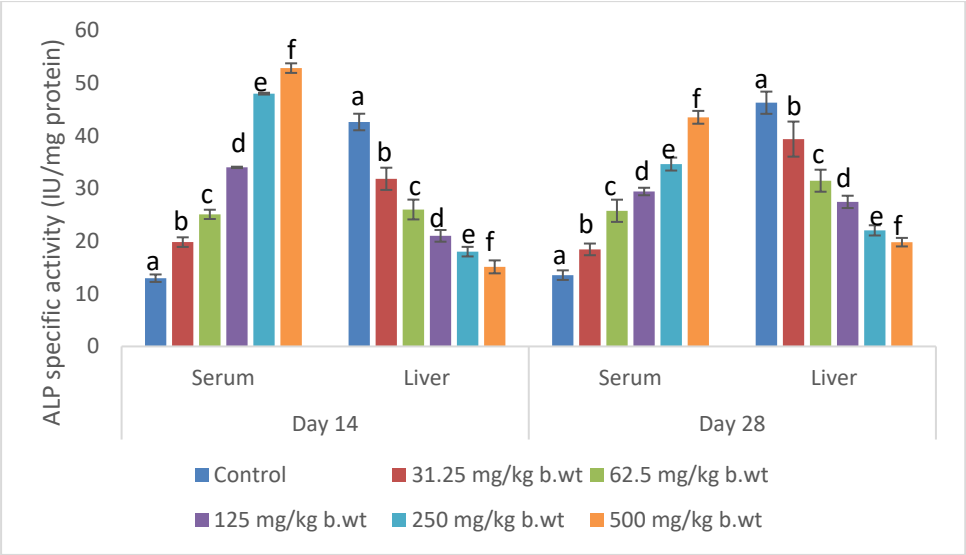

Figure 1. Effects of methanol leaf extract of *C. violaceum* on alkaline phosphatase activities in serum and liver of mice. Values are means of 5 replicates ± SD. Bars with different letters are significantly different ($p < 0.05$)

After 28 days of extract administration, there was a dose-dependent significant increase ($p < 0.05$) in ALP activities in the serum and a significant decrease in the liver compared to controls (Figure 1).

ALP is primarily located on the hepatocyte membrane and shed into the serum, hence the dose-dependent significant decrease in the activity of ALP in the liver throughout the study period compared to controls (Figure 1) may have resulted from the loss of ALP from the membrane into the serum (Levitt et al. 2022). The corresponding dose specific significant increase in the activity of ALP in the serum (Figure 1) confirms this. The reduction in liver ALP activity would hinder adequate transportation of required ions or molecules across their cell membrane and may lead to starvation of cells (Ayorinde et al. 2008).

γ –GlutamylTransferase (γ-GT)

After 14 days of extract administration, there was a significant increase ($p < 0.05$) in γ-GT activity in the serum at all doses except 31.25 mg/kg b. wt compared to controls (Figure 2). There was also a significant decrease ($p < 0.05$) in its activity in the liver at all doses higher than 31.25 mg/kg body weight compared to controls after 14 days of administration (Figure 2).

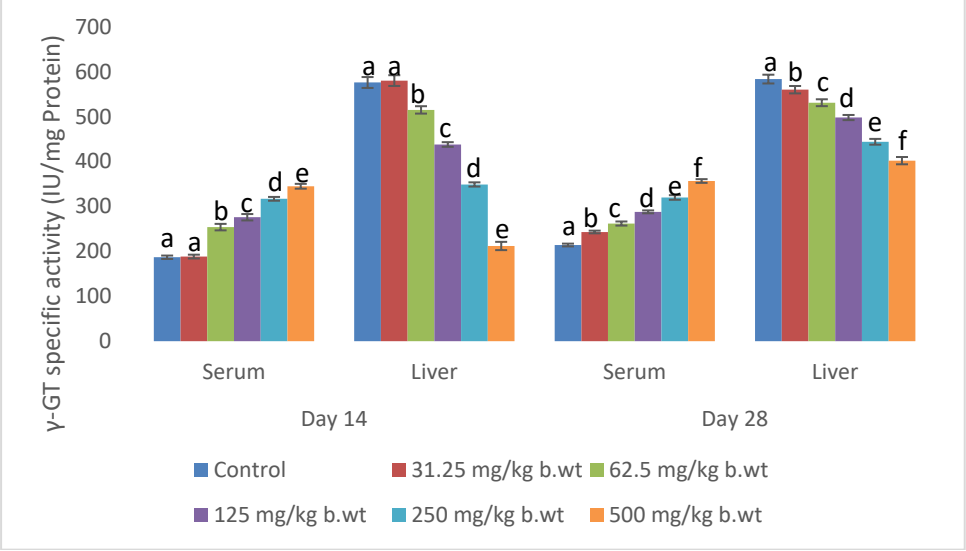

Figure 2: Effects of methanol leaf extract of *C. violaceum* on γ-glutamyltransferase activities serum and liver of mice. Values are means of 5 replicates ± SD. Bars with different letters are significantly different ($p < 0.05$)

After 28 days of administration of extract, there was a significant increase ($p < 0.05$) in γ-glutamyl transferase activity in the serum at all doses and a significant decrease in the liver at all doses compared to controls (Figure 2).
γ-GT is present in the cell membranes of many tissues. It catalyzes the transfer of amino acids across the cellular membrane, and it is involved in leukotriene metabolism. It also plays a major role in glutathione metabolism (Dillon and Miller 2016). γ-GT is the most sensitive enzymatic indicator of hepato-biliary disease because it allows for differentiation of liver diseases from other conditions in which serum ALP activity is elevated since serum γ-GT activity is usually normal in those diseases (Caravaca-Fontán et al. 2017). The decrease in the activity of γ-GT in the liver throughout the study period (Figure 2) may be attributed to leakage of the enzyme from the liver to the serum through altered membranes or because of structural damage done to the liver by the extract as shown by the changes in the architecture of the liver of experimental animals throughout the study period (Plates 1 and 2); this will account for the corresponding increase in the serum ALP activity. These alterations may adversely affect the metabolism of glutathione and resorption of amino acids from the glomerular filtrate and intestinal lumen.

Aspartate Aminotransferase (AST)
There was significant decrease ($p < 0.05$) in AST activity in the liver at the doses of 250 and 500 mg/kg body weight and at 500 mg/kg body weight in the serum compared to controls after 14 days of administration of extract (Figure 3). There was, however, a significant increase ($p < 0.05$) in AST activity in the serum at the other doses compared to control after 14 days of extract administration (Figure 3).

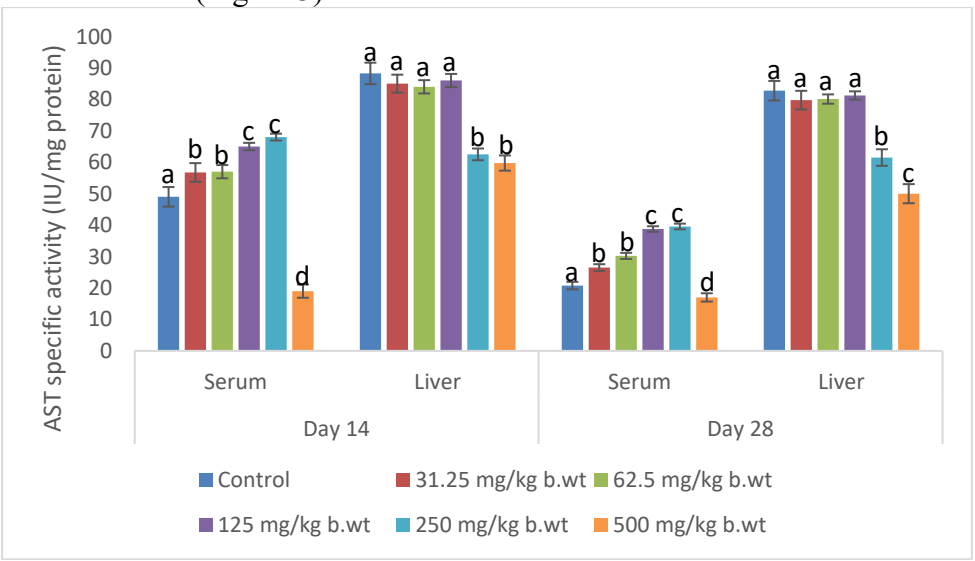

Figure 3. Effects of methanol leaf extract of *C. violaceum* on serum and liver aspartate aminotransferase activity in mice. Values are means of 5 replicates ± SD. Bars with different letters are significantly different ($p < 0.05$)

After 28 days of administration, there was a significant decrease ($p < 0.05$) in AST activity in the liver at doses higher than 125 mg/kg body weight and at the dose of 500 mg/kg in the serum compared to controls. Its activity in the serum at the other doses was significantly increased ($p < 0.05$) compared to controls (Figure 3).

Alanine Aminotransferase (ALT)

There was significant increase ($p < 0.05$) in ALT activity in the liver and serum at the doses of 62.5, 125 and 250 mg/kg body weight after 14 days of extract administration compared to control (Figure 4). There was, however, significant decrease ($p < 0.05$) in its activity in the liver and serum at the dose of 500 mg/kg body weight compared to controls (Figure 4).

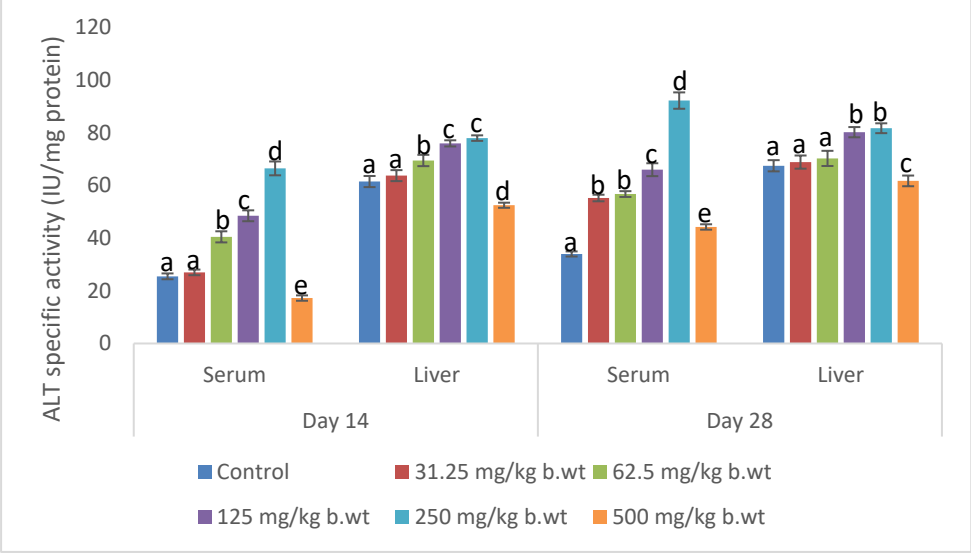

Figure 4. Effects of methanol leaf extract of *C. violaceum* extract on alanine aminotransferase activity in serum and liver of mice. Values are means of 5 replicates ± SD. Bars different letters are significantly different ($p < 0.05$)

After 28 days of extract administration, there was significant increase in ALT activities at the doses of 125 and 250 mg/kg body weight in the liver and at the lower doses in the serum compared to controls (Figure 4). There was a significant decrease ($p < 0.05$) in the activity of the enzyme at 500 mg/kg body weight in the liver compared to control.

AST and ALT are two closely related enzymes of clinical significance in assessment of liver function. They are normally localized within the cells of the liver, heart, kidney, gills, muscles, and others. They are sensitive indicators of hepatocellular damage which can provide a quantitative evaluation of the extent of damage to the liver within limit (Shrestha et al. 2021). The decrease in activity of AST in the liver may have resulted from leakage of the enzyme from the liver at higher doses into the extracellular fluid due to liver cell membrane damage; it may also have resulted from the inactivation of the enzyme *in situ* by extract components at higher doses since its activity in the serum also decreased at the highest dose. Increased inflammatory cells and haemorrhagic necrosis (Plate 2) observed in the liver could have affected its functionality, thus leading to poor plasma clearance of the enzymes. The significant increase in ALT activities in the liver at the doses observed throughout the study period (Figure 4) indicated that the extract may have stimulated increased synthesis of the enzyme *de novo*, which could be an adaptation mechanism by the liver to offset the stress imposed on it by the extract components thus leading to a higher-than-normal activity (Amiragbaya et al. 2021). The reduction in ALT activity at 500 mg/kg body weight in the liver throughout the experimental period suggests that the rate of synthesis must have reduced or it has been inactivated by extract components at the highest dose since the serum activity was also reduced (Figure 4). Injury to the liver intensifies membrane permeability of the parenchyma cell, and consequently, the activity of AST and ALT in serum increases (Wang et al. 2011).

Organ-Body Weight Ratio

The effects of methanol leaf extract of *C. violaceum* on organ-body weight ratios of mice liver after 14 and 28 days of extract administration is shown in Table 3. There was no significant alteration ($p > 0.05$) in the organ-body weight ratios of the liver at all doses administered throughout the study period except at the dose of 500 mg/kg body weight on day 28 which was significantly increased compared to control (Table 3).

Table 3. Effects of Methanol Leaf Extract of *C. violaceum* on Organ-Body Weight Ratios of Mice Liver after 14 and 28 days of Administration

Treatment	Day 14 ($^{x10-2}$)	Day 28 ($^{x10-2}$)
Control	3.74±0.07^{a}	3.61±0.08^{a}
31.25 mg/kg b. wt	4.68±0.02^{a}	3.74±0.07^{a}
62.5 mg/kg b. wt	4.88±0.06^{a}	4.72 ±0.04^{a}
125 mg/kg b. wt	4.23±0.05^{a}	4.77± 0.03^{a}
250 mg/kg b. wt	4.98±0.03^{a}	4.74 ±0.02^{a}
500 mg/kg b. wt	5.09±0.04^{a}	5.99 ±0.06^{b}

Values are means of 5 replicates ± SD. Means in the same column with the same superscripts are not significantly different (p>0.05).

An increase in organ-body weight ratio is an indication of inflammation while a decrease maybe due to cell constriction (Nova 2022). The general absence of any significant alteration on liver-body weight ratios of the mice is an indication that the extract did not adversely affect the size of these organs in relation to the weight of the animals. However, the increased liver-body weight ratio at the dose of 500 mg/kg body weight on day 28 (Table 3) suggests inflammation of the liver because of prolonged extract ingestion.

Histopathological Studies

Histopathological investigation of the liver was done for all experimental groups on days 14 and 28 after extract administration. There was a progressive vacuolar degeneration in the liver of mice at all doses on day 14 compared to control (Plate 1). There was progressive vacuolar degeneration in the liver of mice administered 31.25 and 62.5 mg/kg body weight extract on day 28 compared to control (Plate 2). The animals administered 125 mg/kg body weight of extract had mild vacuolar degeneration of the liver with focal areas of lobular lymphocytic infiltration compared to control (Plate 2). The mice treated with 250 mg/kg body weight of extract had moderate vacuolar degeneration of the liver with focal necrosis of hepatocytes (Plate 2); while animals administered 500 mg/kg body weight extract had extensive haemorrhagic necrosis of the liver compared to control (Plate 2).

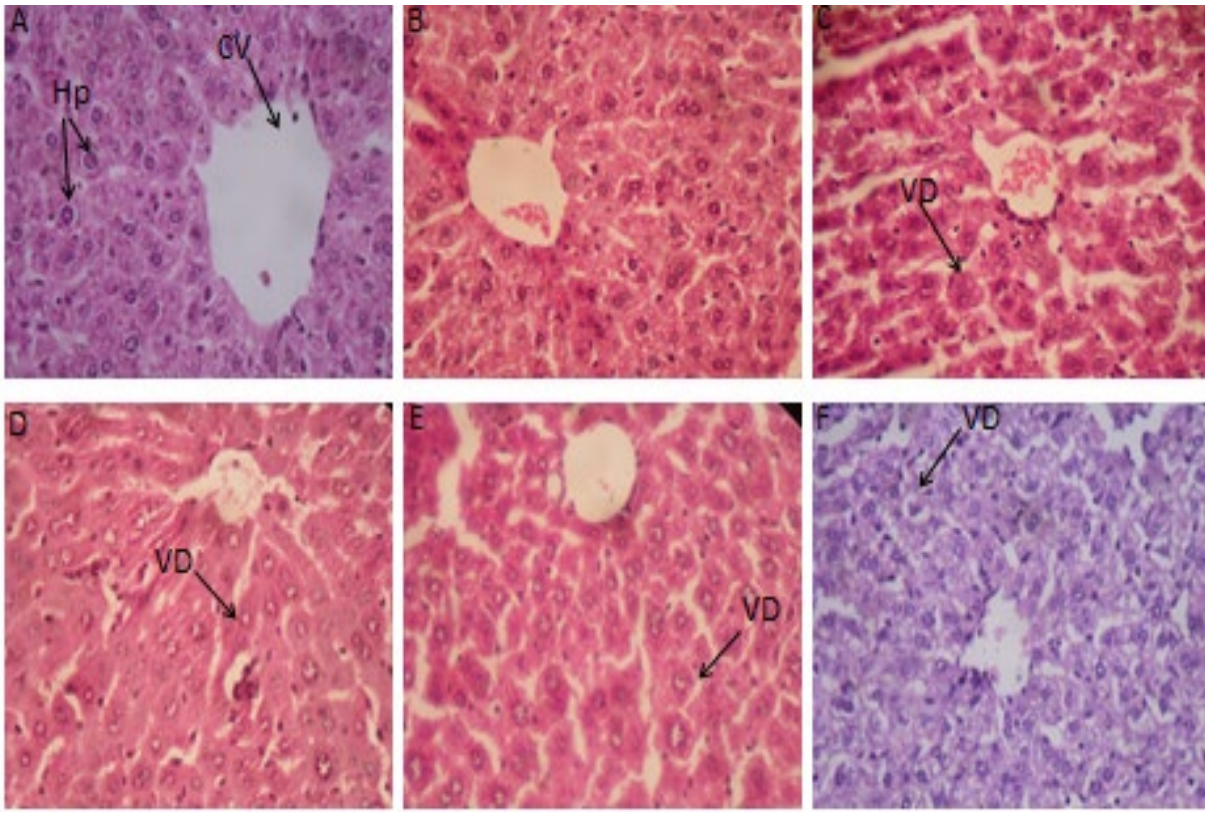

Plate 1. Photomicrographs of the livers of mice administered various doses of methanol leaf extract of *C. violaceum* for 14 days. A, B, C, D, E and F: Control, 31.25, 62.5, 125, 250 and 500 mg/kg b. wt respectively (H and E x400). CV=Central vein, VD=areas with vacuolar degeneration, Hp=normal hepatocytes

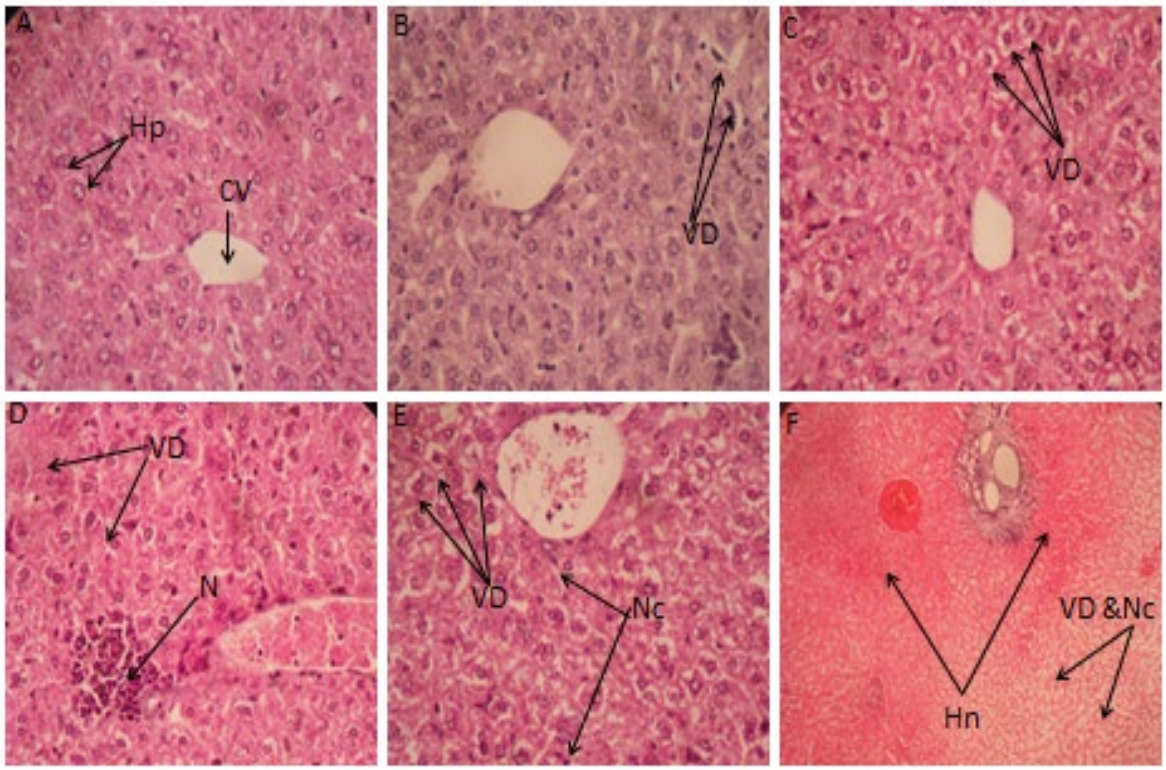

Plate 2. Photomicrographs of the livers of mice administered various doses of methanol leaf extract of *C. violaceum* for 28 days. A, B, C, D, E and F: Control, 31.25, 62.5, 125, 250 and 500 mg/kg b. wt respectively (H and E x400). CV=Central vein, VD=areas with vacuolar

degeneration, Hp=normal hepatocytes, N= neutrophils, Nc=necrosis, Hn=haemorrhagic necrosis.

The measurement of biomolecules in serum and tissue homogenates can indicate tissue damage before it becomes apparent in histopathological screening. The changes in the normal architecture of the liver at higher doses and the presence of inflammatory cells, mainly neutrophils (Plates 1 and 2) suggest that the extract had adverse effects on structure and function of the liver. Neutrophils are characteristically present in the early stages of inflammation (Margraf et al. 2022). There was also degeneration and hemorrhagic necrosis of hepatocytes at higher doses suggesting the toxicity at these doses over a long period of usage.

Conclusions

The results of this study showed that the administration of methanol leaf extract of *C. violaceum* adversely affect the normal architecture, synthetic and secretory functions of the liver of experimental animals. This effect was more pronounced at higher doses of the extract when given for a longer period. Therefore, caution should be exercised when using the decoction of the leaves of this plant especially in large quantities and/or for prolonged periods as this may predispose to adverse effects on the liver.

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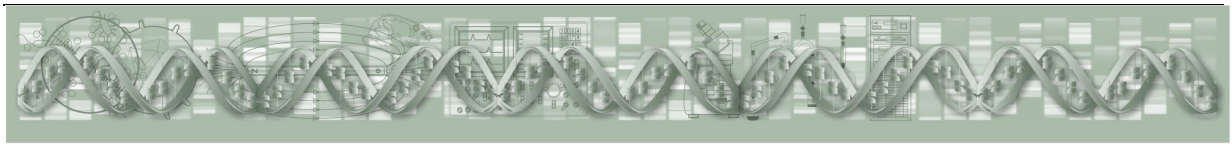

ANALGESIC AND ANXIOLYTIC PROPERTIES OF AQUEOUS EXTRACT OF BARK FROM TRUNK OF *DIOSPYROS MESPILIFORMIS* (EBENACEAE) ON ARTHRITIS INDUCED IN MICE

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Abstract

Arthritis is an inflammatory disease that affects the joints. Patients suffering from chronic pain are anxious, which contributes to reducing the quality of life. The development of analgesic compounds with anxiolytic properties could prove to be of great interest for the treatment of chronic pain. The objective of the present study was to evaluate the analgesic and anxiolytic properties of the aqueous extract of the bark of the trunk of *Diospyros mespiliformis* in arthritic mice. Arthritis was induced by injection of 1% formaline into the left hind paw of the animals. Inflammatory pain and comorbid anxiety were tested using a hotplate (55 ± 0.5 °C) and labyrinths (Dark and Light and the open arena), respectively. The aqueous extract of *Diospyros mespiliformis* reduced the inflammatory process by inhibiting the edema of the legs of animals to a maximum percentage of 63.63 % (minimum of 8.82%) as well as the significant increase ($p < 0.001$) of the threshold of nociception at the dose of 100 mg/kg. A significant increase ($p < 0.001$) in the time spent in the lighted compartment alongside the decrease in the time spent in the dark environment was observed with the two-compartment maze. In the open arena the time spent in the central plaza significantly ($p < 0.001$) increased compared to the time spent on the edge. In addition, a significant decrease ($p < 0.01$) in the frequency of grooming and training was observed. The aqueous extract of the bark of the trunk of *Diospyros mespiliformis* displayed beneficial effects on pain and anxiety, justifying it traditional use for the management of arthritis.

Keywords: analgesic, anxiolytic, *Diospyros mespiliformis*, arthritis.

Introduction

Arthritis is an inflammatory disease of the joints that can affect any joint in the body (Jennifer 2018). It is a bilateral disease mainly localized in the ankles, characterized by continuous swelling around the joint, pain, synovial hyperplasia, pannus formation and morphological changes. These symptoms can lead to severe disability and a poor, unenviable quality of life (Foyet et al. 2015). Arthritis is a very common disease around the world, with more than 91 million people living with it in the United States (Arthritis Foundation 2019). The main clinical symptom of arthritis is pain leading to limited mobility (Vincent et al. 2010), fatigue, impaired muscle strength, muscle weakness, and a change in gait (Abbott et al. 2017, Rice et al. 2015).

Persistent pain leads to anxiety-type mental disorders in some patients with arthritis (Duica 2016). About 40% of patients with chronic arthritis pain are anxious (Twillman 2007). Anxiety is more common in people with any form of arthritis with increased pain sensitivity (Axford et al. 2019, Sharma et al. 2016). The pain and anxiety caused by arthritis should be considered at the same time (Hermans et al. 2012). Thus to fight against arthritis pain and comorbid mental disorders, drugs such as cyclooxygenase (COX) inhibitors are often used in combination with compounds having anxiolytic effects. But such a combination therapy has limits because of the interaction between pharmacological compounds which constitute this treatment and very considerable side effects such as digestive damage (peptic ulcers, stenosis, perforations), renal insufficiency and hepatitis and even cardiac complications (Yougbaré-ziébrou et al. 2016, Soubrier et al. 2013). However, it is difficult to find conventional medications that work to reduce both pain and anxiety without serious risk. It is therefore important to develop alternative analgesic therapies with anxiolytic properties with a limited side effect profile, beneficial for patients suffering daily from chronic pain and associated psychopathologies, such as anxiety.
In recent decades, a lot of research has focused on the valuation of traditional medicine with a view to verify the effectiveness of the substances used and establish scientific rules for their use (Cheriti et al. 2016). *Diospyros mespiliformis* is a plant of the Ebenaceae family, well known in Central Africa for its fruits which are highly appreciated by the population. Commonly known as African ebony, it is registered in the Cameroonian pharmacopoeia and used in traditional medicine for the treatment of arthritis pain and anxiety (Arbonnier 2008). But the population, although they find relief thanks to this treatment, do not know much about the doses and the dosage of this drug, which should not be overlooked when taking drugs. This study was conducted to assess the beneficial effect of different doses of the aqueous extract of the bark of the trunk of *Diospyros mespiliformis* on joint pain and comorbid anxiety disorders.

Material and Methods

Chemicals

To induce peripheral inflammation, mice were injected with formaline (Sigma-Aldrich, St. Louis, MO, USA) subcutaneously under the fascia of animal right paw. To assess changes in paw volume during inflammation, animal were injected in the left hind paw with 0.04 mL of formaline. Control animals received orally distilled water. The anxiolytic and analgesic drugs used were: diazepam, diclofenac (all from Sigma-Aldrich) respectively. Group tested included *Diospyros mespiliformis*.

Phytochemical analyses

Qualitative phytochemical investigations of *Diopyros mespiliformis* aqueous extract were performed for flavonoids, saponins, phenols, lipid, tannins and glycoside cardiac using standard methods previously described Trease and Evans. 1980.

Preparation of plant material

The plant material *Diospyros mespiliformis* collected in the Maroua zone (Region, Far North, Cameroon) (N10°36′45.234″ and E14°16′43.08″) in June 2020. The plant was authenticated at the herbarium of the School of Fauna of Garoua, Cameroon by a reference sample deposited at number HEFG / 01404.
Diospyros mespiliformis was cut into small pieces and dried in the shade then reduced to a very fine powder. Three hundred grams (300 g) of powder was boiled in 2 liters of distilled water for 15 minutes. The solution was filtered using coffee filter paper (pore diameter 20 µm) from BELLE France (Lyon, France) and then evaporated in an oven at 50°C temperature of evaporation.

Animal and experimental design

Animal material: Adult *Mus musculus* Swiss strain mice of both sexes weighing 25 ± 5g and aged 10 ± 2 weeks at the start of the experiment were used. The animals were kept in a room at room temperature in cages lined with litter before and during the period of the experiment. The mice had free access to tap water and standard diet.
Thirty-five mice were distributed into 7 groups of 5 mice each without a distinction of sex (Table 1). All treatments were administered orally thirty minutes before formaline induction (day 0), then the animals were treated daily for up to the 10th day.

Table 1. Grouping of animals

Groups	Administered substance	Doses	Route of administration
Normal	Distilled water	10 (mL/kg)	oral
Negative Control	Distilled water + formaline 1 %	10 (mL/kg)	oral
Positive control	Diclofenac+ formaline 1 %	5 (mg/kg)	oral
Positive control	Diazepam	2 (mg/kg)	oral
Treatment	Aqueous extract+ formaline 1 %	100 (mg/kg)	Oral+ subcutaneous
Treatment	Aqueous extract+ formaline 1 %	200 (mg/kg)	Oral+ subcutaneous
Treatment	Aqueous extract+ formaline 1 %	400 (mg/kg)	Oral+ subcutaneous

Induction of arthritis by formaline 1%
To induce inflammatory arthritis we followed the method by injecting a 1% formaline solution (0.04 mL) under the plantar fascia of the left hind paw of the mouse described by Rahmani et al. (2016) after fasting animals for 17 hours with free access to water. The formaline injection (0.04 mL / mouse; 1%) was performed twice, one in the first day and the other in the third day of the experiment. Treatments were started 30 minutes after induction of arthritis and continued throughout the day until the end of the experiment at doses: 100, 200 and 400 mg/kg of AEDM (aqueous extract of *Diospyros mespiliformis*). The negative control group was treated with distilled water (10 mL/kg) while the positive control groups were treated with diclofenac and diazepam respectively for arthritis and anxiety. The animals' body weight was taken daily using an electronic scale.
The evolution of the edema was followed by measuring the diameter of the edematous paw (mm) of each animal every day throughout the period of the experiment using a digital electronic caliper (precision 0.03 mm). Edema in different groups of animals was determined by the following formula:

$$\mathbf{\Delta E = Ej - E0}$$

ΔE = the difference in edema between j0 and jx
E0 = the initial thickness (mm) of the left paw (before the injection of formaldehyde)
Ej = the thickness of the left paw (mm) at day "j" after the injection of formaldehyde
The percentage of inhibition "% Inh" was calculated by the following

$$\mathbf{\%\ Inh = 100\left[1 - \frac{\Delta ET}{\Delta EC}\right]}$$

ΔEt = represents the difference in edema between j0 and jx of the left paw of the treated mouse
ΔEC = represents the difference in edema between d0 and jx of the left paw of the untreated mouse.

At the end of the experiment, all the animals were sacrificed by cervical dislocation. The blood of each animal was collected in an anticoagulant tube (heparin) and then centrifuged at 3000

round/min for 15 minutes at 4 ° C. The sera obtained were collected in microtubes for the assay of the C-reactive protein (CRP). The livers were isolated and then homogenized in a phosphate buffer (0.15M, pH = 7.4) (Zuo et al. 2014).
The supernatant was used for evaluation of some oxidative stress parameters: Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). The hind legs were removed and fixed in 10 % formalin solution for histological studies.

ANXIETY TEST

Lighted and Dark Compartment Maze Test

The device consisted of a light / dark box (45 x 27 x 27 cm) and composed two chambers connected by an opening (7.5 x 7.5 cm) located at ground level in the center of the wall separating the two chambers.
The small chamber (18 x 27 cm) was painted black (dark room) and the larger room (27 x 27 cm) was painted white (bright room). The parameters taken into account were: latency time, time spent in the lighted compartment, time spent in the dark compartment. Each mouse was placed in the center of the light chamber back to the dark room and allowed to explore both compartments of the device for 5 minutes. After 5 minutes, the animal was removed from the device and the device cleaned with a 70% ethanol solution and allowed to dry between tests.

Open arena test

It is an open space arena, square in shape (72 × 72 cm) and 36 cm high. Visible red lines were drawn on the floor using a marker (Foyet et al. 2012). These lines have the role of delimiting a central (ZC), intermediate (ZI) and peripheral (ZP) zone near the wall. The time spent in the center and on the edge, the training (reaning), the grooming, the number of lines crossed were the parameters taken into account during the tests. After each test, the mouse was removed and returned to its cage. The entire maze floor was cleaned with a 70% ethanol solution after each test and allowed to dry between tests.

Hyperalgesia test

This test consists of a hot plate apparatus maintained at a temperature of 55 ± 0.5 ° C on which the mice were placed for the test (Foyet et al., 2015). The pain threshold was determined by the latency of the nociceptive response (reaction time for the animal to lick the paw or jump off the hotplate) with a maximum cut-off time of 15 s for each animal (Foyet et al. 2015).

Evaluation of the antioxidant activity *in vivo* of the aqueous extract of the bark of *Diospyros mespiliformis*

The antioxidant potential of the extract was assessed by estimating Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) in the liver homogenate of according to the methods of Wilbur et al. in 1949, Misra and Fridovish in 1972, Sinha in 1972 and Ellman in 1959 respectively.

Histological study

For microscopic evaluation, the investigated organs were dehydrated and paraffin-embedded for microscopic examination in accordance with routine laboratory procedures. Paraffin sections of 5 µm were prepared and stained with haematoxylin and eosin for histological examination.

Statistical analyzes

Results were expressed as the mean ± standard error of the mean (ESM) for each group. Number per group = 5. The one way analysis of variance test (Anova) was used followed by the Student Newman Kells post test to compare the values with each other. The results were considered to be significantly different for $p < 0.05$.

Results

Qualitative Phytochemistry

Phytochemical screening of the aqueous extract of the bark of the trunk of *Diospyros mespiliformis* revealed the presence of several bioactive compounds (Table 2).

Table 2. Phytochemical of the aqueous extract of *Diospyros mepiliformis*

Compound class	Alcaloids	Flavonoids	Tanins	Saponins	Terpenoids	Sugar	Quinons	Coumarins
Observation	+	+	+	+	-	+	+	+

+ = presence - = absence

Effect of *Diospyros mespiliformis* aqueous extract on weight gain in mice

A decrease in body weight was noted in all experimental groups except the normal group after the days following formaline injection. This decrease was observed in the negative group from the fourth day and the weight of the animals continued to decrease until the end of the experiment. The difference was only significant ($p < 0.01$) in the negative compared to the normal control. On the other hand, for the treated groups, weight recovery was noted during the last days of the experiment (Figure 1). Treatment of the mice with the aqueous extract of *Diospyros mespiliformis* did not influence the body weight of the animals.

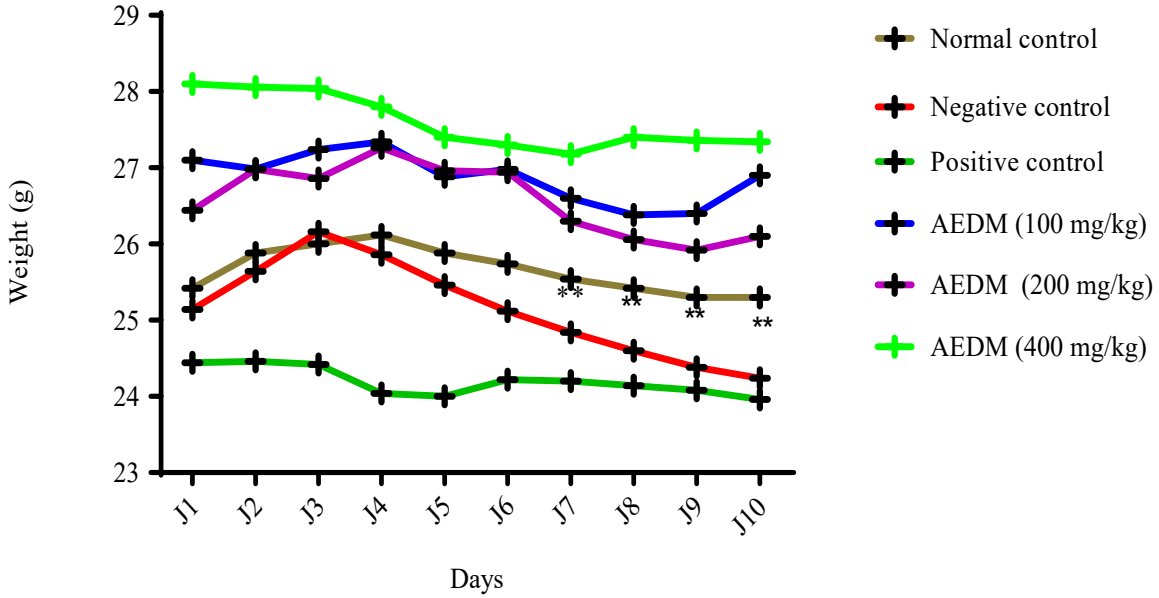

Figure 1. Effect of aqueous extract of *Diospyros mespiliformis* aqueous extract on weight gain in mice

Values represented as means ± SEM (n = 5 for each group). **p<0, 01 for comparison between normal group, one-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used.

AEDM: Aqueous extract of *Diospyros mespiliformis.* J = day

Effect of aqueous extract of *Diospyros mespiliformis* on the course of paw edema in animals

The evolution of edema (ΔE) of the inflamed paw during the experimentation period at D2, D4, D6, D8 and D10 is illustrated in Figure 2 below. These data show a reduction in paw edema

represented by the difference between the diameters of the paws - inflamed and non-inflamed (ΔE) in all treated groups compared to the negative control group. Treatment with diclofenac (5 mg / kg) significantly ($p < 0.001$) reduced the edema of the paws of the animals on days (D6, D8, D10) of treatment compared to the negative group. The aqueous extract of *Diospyros mespiliformis* also significantly ($p < 0.001$) reduced paw edema on days D8 and D10 at AEDM doses 100 mg/kg, 200 mg/kg compared to the negative control. On the 6th day (D6) of the experiment, the extract also significantly reduced edema ($p < 0.001$) (AEDM 100 and 200 mg/kg); ($p < 0.01$) (AEDM 400 mg/kg) compared to the negative control.

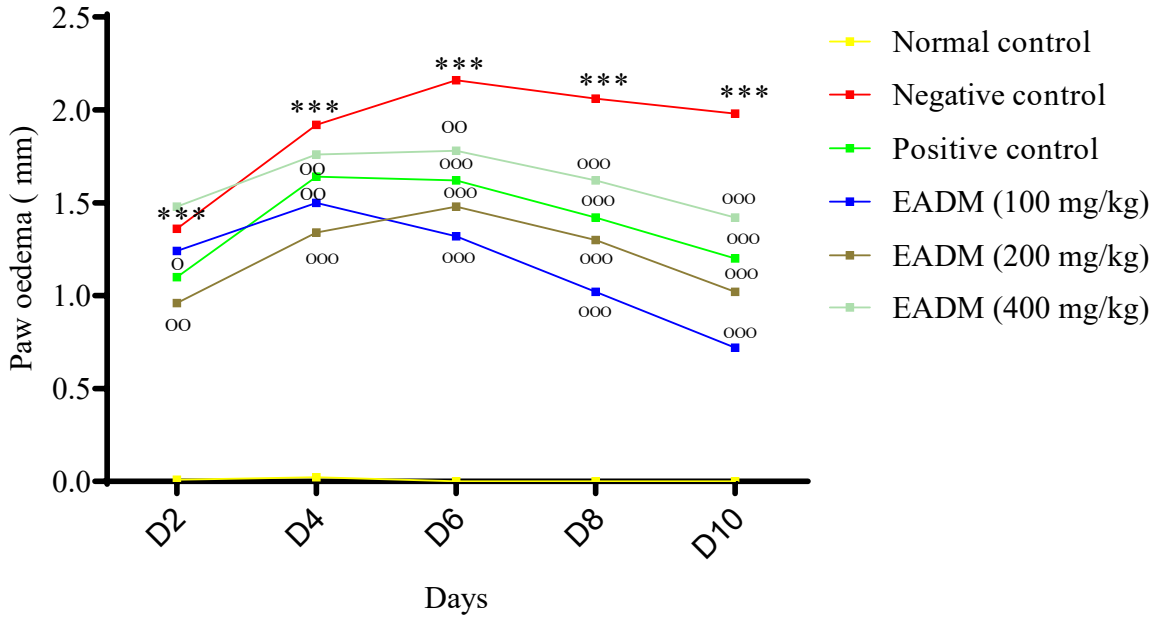

Figure 2. Effect of aqueous extract *of Diospyros mespiliformis* on the course of paw edema in mice

Values represented as means ± SEM (n = 5 for each group). ΘΘ P < 0.01 ΘΘΘP < 0.001 and vs control group. For comparison between groups, one-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of *Diospyros mespiliformis* aqueous extract on percent inhibition of paw edema in mice

The paw edema of the mice was significantly but unevenly inhibited in the treated groups on the last three days of treatment. The diclofenac group reached only 39.39% on the last day of the experiment. On the other hand, the groups which received the plant extract showed a percentage inhibition of 63.63%, 48.48 and 28.29% respectively at the doses of 100, 200 and 400 mg/kg on the last day of the experiment (Table 3). The maximum inhibition (63.63%) of the diameter of the edematous legs of the animals at the end of the experiment was recorded in the group treated with the plant extract at the dose of 100 mg/kg. This dose is considered the most effective.

Table 3. Effect of aqueous extract of *Diospyros mespiliformis* on percent inhibition of edema in mouse paws

Group/Dose (mg/kg)	Number	Percentage inhibition (% inh) of paw edema				
		J2	J4	J6	J8	10
DCF 5 mg/kg	5	19.11	14.53	25.00	31.06	39.39
AEDM 100 mg/kg	5	8.82	21.87	38.88	50.48	63.63
AEDM 200 mg/kg	5	29.41	30.20	31.48	36.89	48.48
AEDM 400 mg/kg	5	8.82	8.33	17.59	21.35	28.29

DCF: diclofenac, **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of *Diospyros mespiliformis* extract on plasma concentration of C-reactive protein (CRP)

The results showed a difference in concentration between the mean values of CRP in the different groups of experimental animals. A significant increase ($p < 0.05$, $p < 0.001$, respectively) was observed in the animals treated with the plant extract at the dose of 400 mg / kg and in the negative control group compared to the normal control. Doses 100 and 200 mg / kg of plant extract significantly lowered the plasmatic concentration of CRP ($p < 0.001$; $p < 0.01$, respectively) compared to the negative control group (Figure 3).

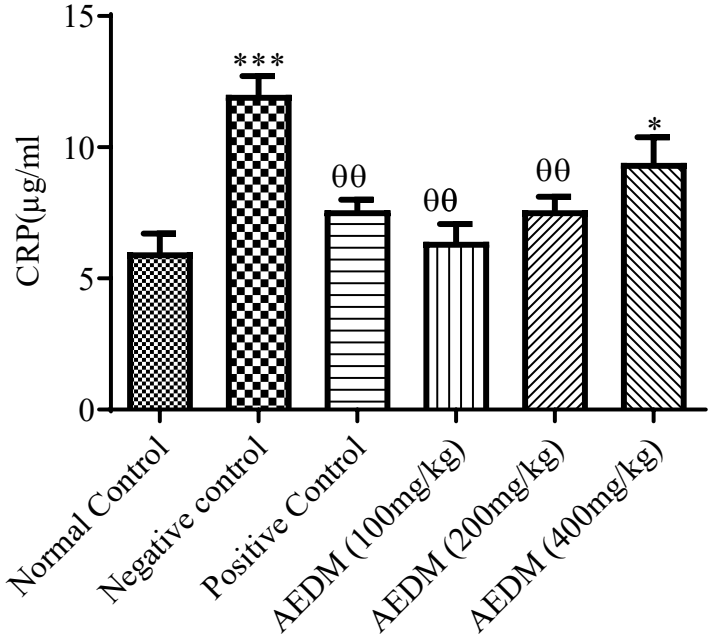

Figure 3. Effect of aqueous extract of *Diospyros mespiliformis* on plasmatic concentration of C-reactive protein

Values represented as means ± SEM (n = 5 for each group). * $p < 0.05$, *** $p < 0.001$ compared to the normal group; θθ $p < 0.01$; θθθ $p < 0.001$ compared to the negative group, one-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of aqueous extract of *Diospyros mespiliformis* on hyperalgesia activity

The reaction time of the mice to the pain test showed a significant decrease ($p < 0.01$) in the negative control animals compared to the normal control. On the other hand, in the groups treated with the plant extract, we observed a significant increase ($p < 0.001$) in the latency time at doses of 100 and 200 mg / kg. The lag time increased significantly ($p < 0.001$) in animals treated with diclofenac 5 mg / kg compared to the normal control. However, a significant increase ($p < 0.001$) in mouse reaction time was observed at all doses of the extract and diclofenac compared to the negative control (Figure 4).

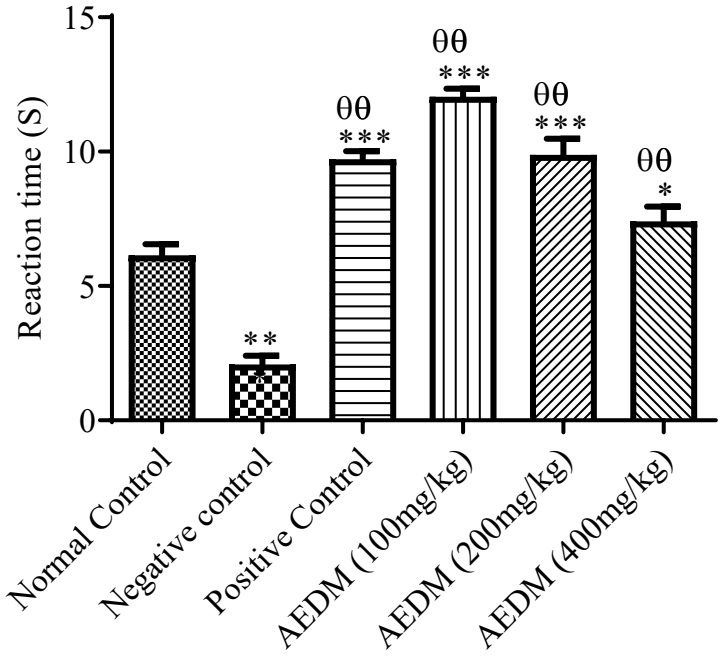

Figure 4. Effect of aqueous extract of *Diospyros mespiliformis* on hyperalgesia activity
Values represented as means ± SEM (n= 5 for each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the normal control; θθ $p < 0.001$ compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of the aqueous extract of *Diospyros mespiliformis* in the light and dark compartment box test

The results showed no significant difference between the negative control and the normal control (Figure 5). In contrast, treatment with diazepam (2 mg / kg) resulted in a significant increase ($p < 0.001$) in latency time compared to normal and negative controls. AEDM was also significantly ($p < 0.001$) increased by normal and negative controls. But at the dose of 400 mg / kg of extract we observe a significant increase ($p < 0.05$) compared to the normal and negative controls.

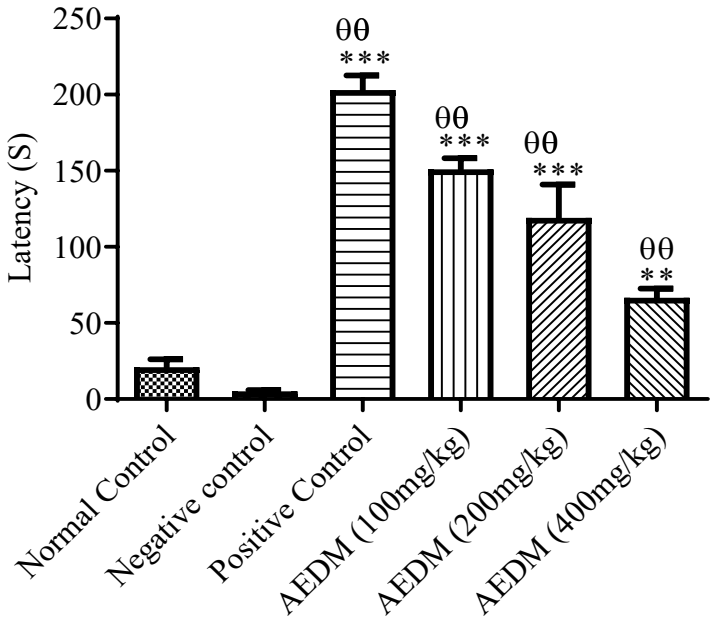

Figure 5. Effect of *Diospyros mespiliformis* on the latency time in the Light and Dark box test Values represented as means ± SEM (n=5 for each group). ** p <0.01, *** p <0.001 compared to the normal group; θθ p <0.01, θθθ p <0.001 compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of *Diospyros mespiliformis* on the time spent in the dark and lighted compartments of the labyrinth

The results of this test show that the mice in the normal control group and in the negative control group prefer the dark environment of the box. On the other hand, the mice which received different treatment prefer the lighted environment. The mice which received the 2 mg / kg dose of diazepam showed a significant increase ($p < 0.001$) in the time spent in the lighted medium coupled with a significant decrease ($p < 0.001$) in the time spent in the dark medium compared to the normal controls and negative control (Figure 6). The same phenomenon was observed in mice given the plant extract at all doses, but only compared to the negative control. The difference in the time spent in the lighted environment of the labyrinth of mice treated with plant extract compared to the normal control shows a significant dose-dependent variation ($p < 0.001$) (AEDM 100 mg / kg); ($p < 0.01$) (EDM 200 mg / kg); ($p < 0.05$) (AEDM 400 mg / kg). As regards the time spent in the dark medium, a significant decrease ($p < 0.001$) in the time is noted in the treated batches (DZP 2 mg / kg, AEDM 200 mg / kg). At a dose of 400 mg / kg, the extract decreased significantly ($p < 0.05$) compared to the negative control group.

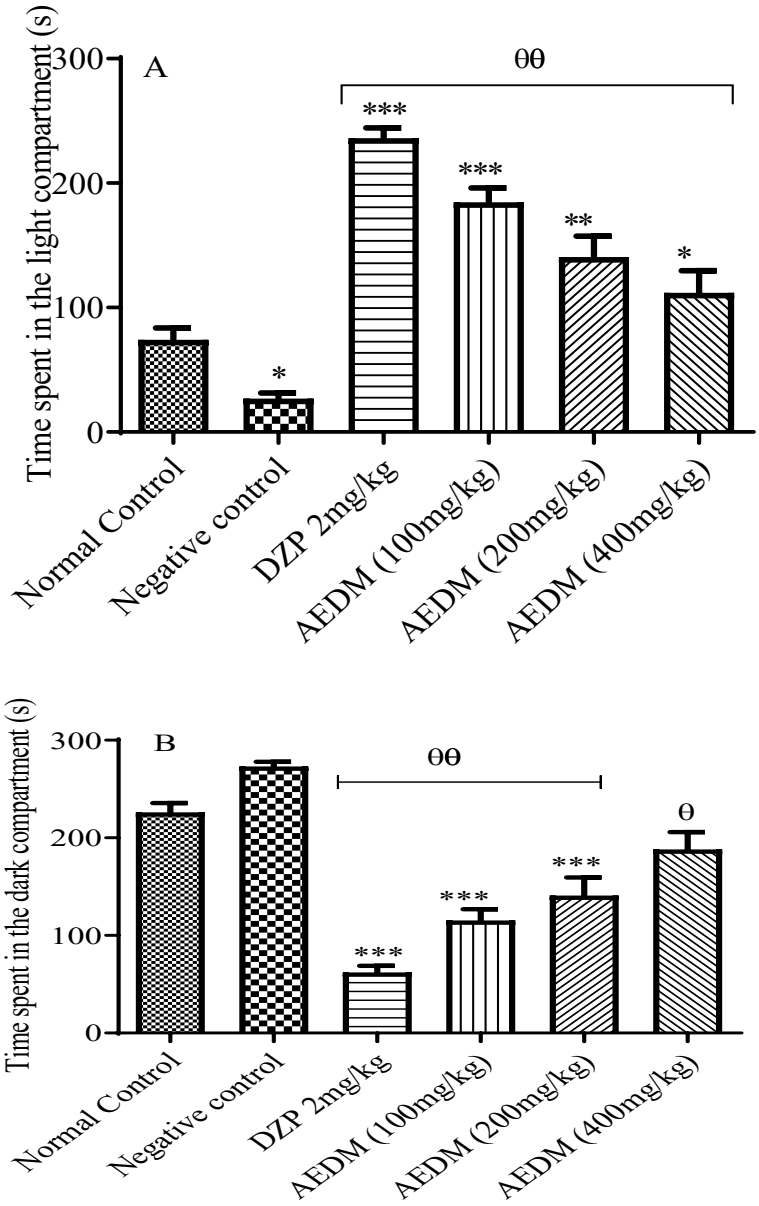

Figure 6. Effect of *Diospyros mespiliformis* on time in the light (a) and dark (b) compartments of the labyrinth

Values represented as means ± SEM (n=5 for each group). * p <0.05, ** p <0.01, *** p <0.001 compared to the normal group; θ p <0.05, θθ p <0.001 compared to the negative group. one-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **DZP** = diazepam, **AEDM**: aqueous extract of *Diospyros mespiliformis*

Effect of *Diospyros mespiliformis* aqueous extract in the open arena test

The results showed that the animals that received no treatment spent more time at the edge of the maze and little time in the middle but unevenly. Arthritic mice spend more time at the edge compared to normal mice (267.0 ± 5.657 s> 254.2 ± 5.678 s) (Table 4) and less time in the middle place compared to the latter (33.00 ± 5.657 s < 45.80 ± 5.678 s). Time spent at the center was significantly increased ($p < 0.001$) in animals that received diazepam (2 mg / kg) compared to controls. It also increased significantly ($p < 0.001$) (100, 200 mg / kg); ($p < 0.05$) (400 m / kg) in animals treated with aqueous extract of *Diospyros mespiliformis* compared to normal and negative control groups. Time spent on board was significantly reduced in treated animals (p <

0.001) (DZP 2m g / kg, AEDM 100 mg / kg); ($p < 0.05$) (200 mg / kg, 400 mg / kg). The locomotor capacity of the mice was evaluated by the number of lines crossed in the field of the labyrinth. The animals of the negative control group show a significant decrease ($p < 0.01$) in the number of crossed lines compared to the normal control. The number of crossed lines increased significantly ($p < 0.001$) (100 mg / kg); ($p < 0.01$) (200 mg / kg) compared to normal control in animals treated with AEDM. In addition, the frequency of grooming and rearing differed in the experimental batches. The negative group showed a significant decrease ($p < 0.05$) in the frequency of grooming compared to the normal control. In the treated batches, the reduction in the frequency of grooming was significant ($p < 0.001$) compared to the untreated controls. For rearing, the extract significantly ($p <0.01$) reduced its frequency compared to untreated controls. Treatment of mice significantly ($p < 0.05$) (DZP 2 mg / kg, 400 mg / kg) ($p <0.01$) (100 mg / kg) reduced the defecation spot.

Table 3. Effect of *Diospyros mespiliformis* extract on some parameters evaluated in the open arena box

Groups/ Doses (mg/kg)	Time at the center (s)	Time at the edge	Number of line crossed	Frequency of grooming	Frequency of rearing	Spot of defecation
Normal	45.80 ±5.67	254.20 ± 5.67	32.40 ± 4.13	4.40 ± 0.50	5.20 ± 0.66	1.50 ± 0.23
Negative	33.00 ± 5.65	267.0 0 ± 15.65	15.80 ± 1.35 **	6.60 ± 0.50*	5.60 ± 0.50	2.16 ± 0.31
DZP (2mg/kg)	242.60 ± 16.59*** θθθ	37.40 ± 1.70 *** θθθ	32.40 ± 4.13	1.61 ± 0.51 *** θθθ	2.80 ± 0.86θ	1.00 ± 0.36θ
AEDM (100mg/kg)	190.20 ± 13.84 *** θθθ	133.80 ± 10.29 *** θθθ	63.00 ± 5.06 θθθ	0.80 ± 0.37*** θθθ	1.60 ± 0.50**θθθ	0.67 ± 0.33 θθ
AEDM (200 mg/kg)	139.20 ± 8.73 *** θθθ	174.80 ± 17.88* θ	58.40 ± 5.30 θθ	1.00 ± 0.31 *** θθθ	1.80 ± 0.58**θθ	1.00 ± 0.36 θ
AEDM (400mg/kg)	125.20 ± 27.88 * θθθ	125.20 ± 27.88 * θ	37.20 ± 4.27	1.70 ± 0.35 *** θθθ	2.00± 0.32 **θθ	1.00 ± 0.52

Values represented as means ± SEM (n=5). * $p <0.05$, ** $p <0.01$, *** $p <0.001$ compared to the normal group; θp <0.05, θθ $p <0.01$, θθθ $p <0.001$ compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **DZP:** diazepam; **AEDM**: extract aqueous of *Diospyros mespiliformis*

Effect of the aqueous extract of the bark *of Diospyros mespiliformis* on certain oxidative stress parameters

Effects of the aqueous extract of *Diospyros mespiliformis* on the concentration of Malondialdehyde (MDA) in the liver

The level of MDA was significantly increased ($P < 0.01$) in the negative controls compared to the normal control (Figure 7). In the treated batches, the level of MDA decreased significantly ($P < 0.001$) (AEDM 100 mg / kg, AEDM 200 mg / kg 2 mg / kg); ($P < 0.01$) (AEDM 400 mg / kg) compared to the negative control. This decrease was also significant ($P < 0.01$) (EADM 100 mg / kg and 2 mg / kg); ($P < 0.05$) (200 mg / kg).

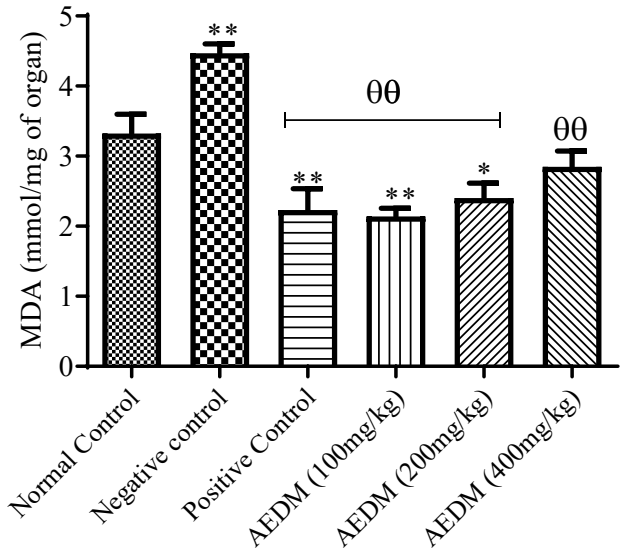

Figure 7. Effects of the aqueous extract of *Diospyros mespiliformis* on the concentration of liver tissue in MDA
Values represented as means ± SEM (n=5). * p <0.05, ** p <0.01, compared to the normal group; θθ p <0.01, θθθ p <0.001 compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: extract aqueous of *Diospyros mespiliformis*

Effects of *Diospyros mespiliformis* aqueous extract on superoxydismutase (SOD) activity in the liver

The results showed a significant increase ($p < 0.05$) in the level of SOD in the group treated with diclofenac (5 mg / kg) compared to the normal control. This rate increased significantly ($p < 0.001$) compared to the negative control. Treatment with aqueous extract of *Diospyros mespiliformis* at all doses significantly ($p < 0.05$) increased SOD activity in liver tissue of animals compared to the negative control.

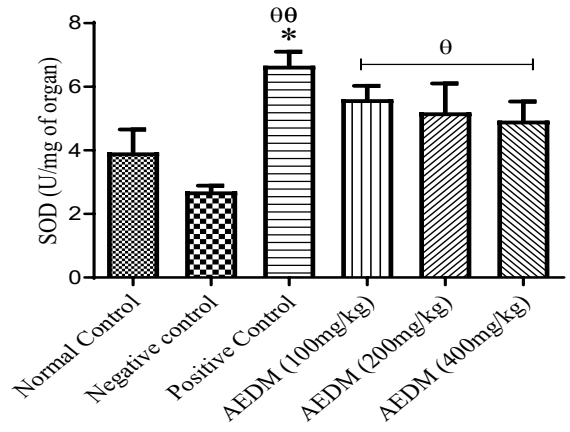

Figure 8. Effects of *Diospyros mespiliformis* aqueous extract on SOD
EADM: extract aqueous of *Diospyros mespiliformis*
Values represented as means ± SEM (n=5). * p <0.05, compared to the normal group; θ p <0.05, θθθ p <0.001 compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used.

Effects of *Diospyros mespiliformis* on catalase activity (CAT)

The level of catalase was significantly ($p < 0.05$) increased in the group treated with diclofenac (5 mg / kg) compared to the negative control. The aqueous extract of *Diospyros mespiliformis* also increased significantly ($p < 0.001$) (AEDM 100 mg / kg); ($p < 0.05$) (AEDM 200 and 400 mg / kg) compared to the negative control.

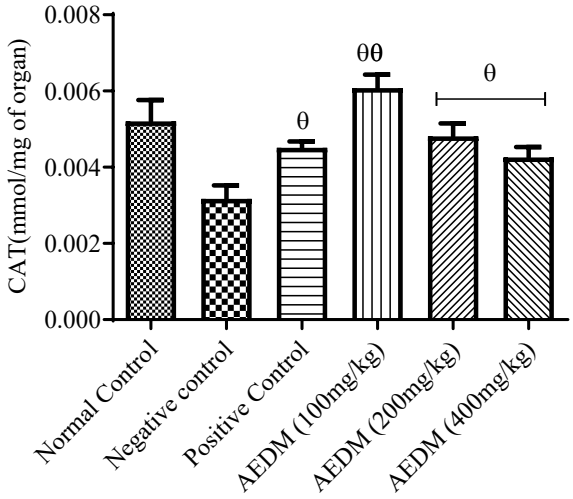

Figure 9. Effects of *Diospyros mespiliformis* on catalase activity

Values represented as means ± SEM (n=5). θ $p < 0.05$, θθ $p < 0.001$ compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: extract aqueous of *Diospyros mespiliformis*

Effects of *Diospyros mespiliformis* aqueous extract on hepatic glutathione (GSH) levels

Diospyros mespiliformis aqueous extract significantly increased glutathione ($p < 0.001$) (AEDM 100 mg / kg); ($p < 0.01$) (DCF 5 mg / kg) compared to the negative control. No significant difference was observed in any treated animals compared to the normal control.

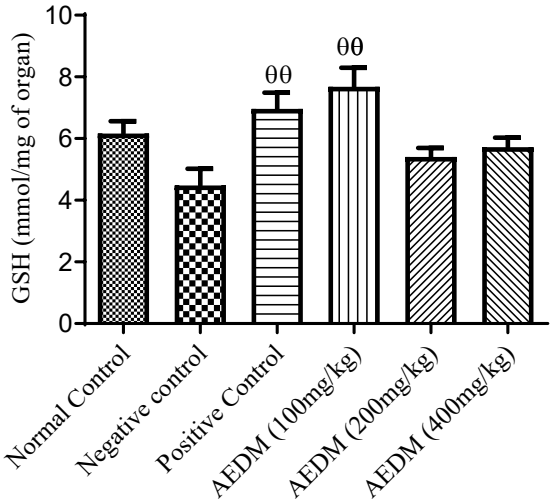

Figure 10. Effects of the aqueous extract of *Diospyros mespiliformis* on the hepatic level of glutathione (GSH)

Values represented as means ± SEM (n=5). θθ $p < 0.01$, θθθ $p < 0.001$ compared to the negative group. One-way analysis of variance (ANOVA) with Newman Kells multiple comparison tests was used. **AEDM**: extract aqueous of *Diospyros mespiliformis*

Effect of the aqueous extract of the bark of *Diospyros mespiliformis* on the histology of the legs

Photomicrographs of the paw of the animals show normal structure of the epidermis and dermis in a normal control group. The negative control animals showed in comparison with the normal control, inflammation and thickening of the epidermis and dermis. An improvement in the structure of the dermis compared to the negative control was observed in the batches treated with the aqueous extract of *Diospyros mespiliformis* and with diclofenac.

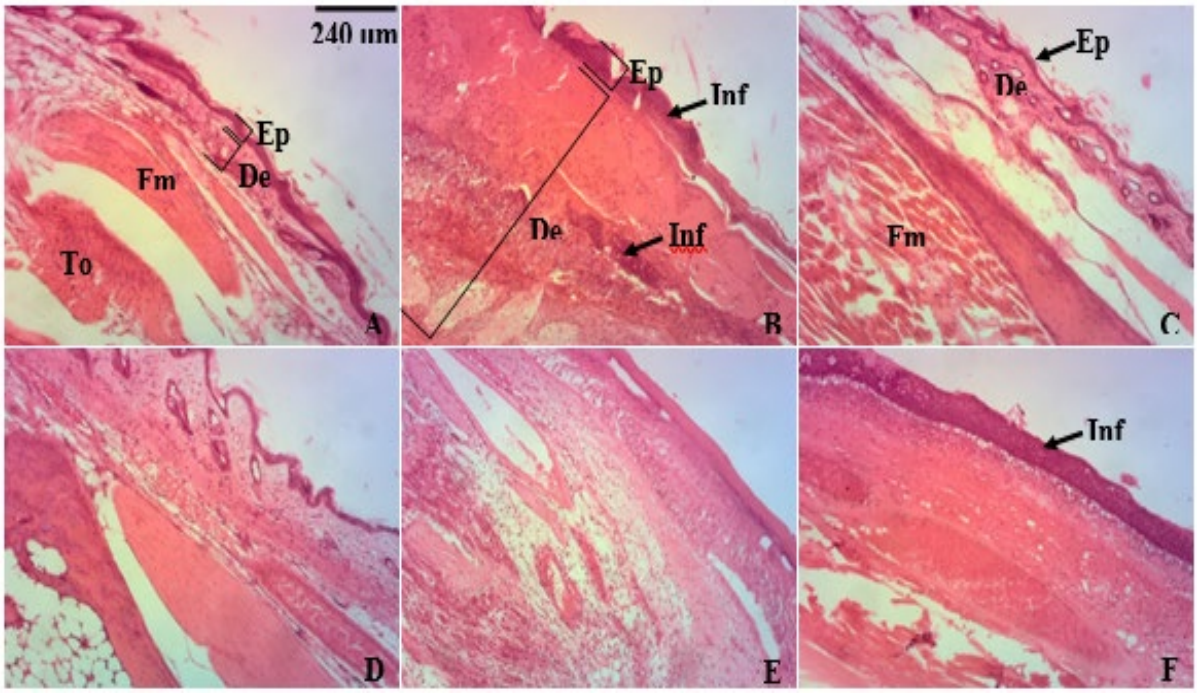

Figure 11. Photomicrograph of the arch of the foot (Hematoxylin-eosin X 40). A: Normal control; B: Negative control; C: diclofenac 5 mg / kg; D: *Diospyros mespiliformis* 100 mg / kg; E: *Diospyros mespiliformis* 200 mg / kg; F: *Diospyros mespiliformis* 400 mg / kg; Ep: epidermis; De: Derma; Fm: Skeletal striated muscle fibers; Inf: Inflammation.

Discussion

The analgesic and anxiolytic properties of the aqueous extract of the bark of the trunk of *Diospyros mespiliformis* were evaluated in arthritis in mice. Injection of a formaline solution into the fascia of the left hind paw of each mouse caused swelling of the paws of the mice. The increased leg size of mice is a sign of a formalin-induced inflammatory reaction leading to edema (Rahmani et al. 2016). This inflammatory response is biphasic, the first phase is due to the release of histamine and during the second phase there is release of serotonin, bradykinin, prostaglandins (Atsang et al. 2014). These inflammation mediators are responsible for vasodilation and increased vascular permeability (Gao et al. 2009, Foughalia 2017). The exudate consequently escapes from the bloodstream to the intestinal medium causing edema and pain (Mansour. 2015). The nociceptive response during inflammation has two phases, the first phase mediated peripherally by the release of chemical mediators, the second neurogen mediated centrally with direct stimulation of the C fiber and the release of substance P (Atsang et al. 2014). Diclofenac is a drug known for its ability to counter the symptoms of the inflammatory reaction (Bektas et al. 2012). It is prescribed for the treatment of arthritis and muscle pain (Akinnawo et al. 2017). The administration of this drug inhibits cyclooxygenase 1 and 2 (cox-1 and cox-2) which leads to a decrease in prostaglandins and thromboxanes thus

leading to a decrease in inflammatory effects (Marchlewicz et al. 2016). The aqueous extract of *Diospyros mespiliformis* significantly ($p < 0.001$) reduced inflammatory edema compared to the negative control with a percent inhibition of 63.63, 48.48 and 28.29% respectively at doses of 100, 200 and 400 mg / kg. This reduction was coupled with a significant ($p < 0.001$) decrease in plasma C-reactive protein (CRP) concentration. CRP is a hepatic protein released during the inflammatory response. It is an early, sensitive and specific marker of the inflammatory reaction increasing in proportion to its intensity (Povoa 2002). EADM also significantly increased the pain threshold. The aqueous extract of *Diospyros mespiliformis* may have an inhibitory effect on cyclooxygenase and lead to a decrease in prostaglandins. These effects are thought to be due to the presence of bioactive compounds such as alkaloids, flavonoids, tannins, coumarins and glucosides which are endowed with significant analgesic and anti-inflammatory properties (Tunalier et al. 2007, Batista et al. 2009). This is because flavonoids, coumarins and tannins are phenolic compounds that act on inflammation by inhibiting enzymes involved in the arachidonic acid mechanism and enzymes that generate reactive oxygen species as well as inhibition of NFKB transcription factors. Alkaloids, for their part, have anti-inflammatory properties by direct inhibition of phospholipase A2 (Lamnaouer 2008). On the other hand, people with persistent arthritis pain exhibit similar patterns of avoidance activity and anxiety which are a very common comorbidity (LE Simons et al. 2012). This worrying clinical situation makes it difficult for the attending physician to select the pharmacological approach to be favored for effective treatment (Rabenda et al. 2005). However, in addition to the analgesic effect, the anxiolytic activity of AEDM was evaluated. The test in the two-compartment labyrinth provides information on the emotional state of rodents according to their preference for the dark compartment considered as non-anxiety-inducing and the illuminated anxiety-producing compartment (Ramos et al. 2008). The aqueous extract of *Diospyros mespiliformis* reduced the time spent in the dark compartment compared to untreated controls at doses of 100, 200 and 400 mg / kg. Treatment with diazepam (2 mg / kg) significantly ($p < 0.001$) increased the time spent in the lighted compartment compared to untreated controls. Likewise, AEDM significantly increased ($p < 0.001$) at all doses the time spent in the illuminated compartment compared to the negative control. But to degrees of dose-dependent significance ($p < 0.05$) (400 mg / kg); ($p < 0.01$) (200 mg / kg); ($p < 0.001$) (100 mg / kg) compared to the normal control. Alongside the time spent in the two compartments of the box, the normal control animals spontaneously enter the dark compartment in order to take refuge in the dark. Diazepam (2 mg / kg) significantly ($p < 0.001$) increased latency compared to two controls. AEDM also increased significantly ($p < 0.001$) (100 and 200 mg / kg); ($p < 0.01$) (400 mg / kg) compared to the negative and normal control. Anxious people are generally driven by a feeling of fear, doubt and avoidance (Sandeep et al. 2017). In fact, the more time the animal spends in the dark environment, the more anxious it is and the more it spends in the lighted environment, the less anxious it is. AEDM could have an anxiolytic effect given the behavior of the animals observed in the maze. The effect of the plant was confirmed by a second test in the open field box "The Open field" which made it possible to assess the level of anxiety in the animals by comparing the time spent in the central place and in the middle edge of the box, the number of lines crossed, the frequency of training and grooming and the defecation spot in the different groups. The increase in locomotor capacity and general activity in the central zone is interpreted as an anxiolytic effect (Prut et al. 2003). The time spent in the central place by animals treated with diazepam (2 mg / kg) increased significantly ($p < 0.001$) compared to control mice. The time spent in the central plaza by the animals treated with the aqueous extract of *Diospyros mespiliformis* also increased significantly ($p < 0.001$) at the doses of 100, 200 and 400 m / kg ($p < 0.05$). AEDM significantly improved locomotor capacity in animals ($p < 0.001$) at the dose of 100 mg / kg. The frequencies of grooming and rearing also express a state of anxiety. However, *Diospyros mespiliformis* extract significantly reduced ($p < 0.001$) the number of

grooming and rearing compared to control animals. These results suggest that the aqueous extract of *Diospyros mespiliformis* improves the state of anxiety. This action could be linked to the presence in the extract of phenolic compounds including flavonoids, tannins known for their ability to improve mood and the disorder that occurs in the central nervous system (Bibi et al. 2017). Patients with rheumatoid arthritis show significant variations in liver enzyme activity (Bihani et al. 2014) with liver and kidney damage. The inflammatory reaction increases free radicals such as O_2^-(superoxide anion) hydrogen peroxide (h_2o_2) and hydroxyl radical (OH) responsible for oxidative stress and which can damage DNA, proteins and lipids (Vital et al. 2013). In the body, SOD prevents the accumulation of O_2 and transforms it into H_2O_2 and O_2 by catalase (Rabaud et al. 1997). Determination of the oxidative stress parameters revealed a significant increase ($p < 0.001$) in the level of MDA in the negative control group compared to the normal control group. MDA is a biomarker of lipid peroxidation of membranes (Foyet et al. 2019). The increased concentration of MDA in liver tissue in the negative control group suggests that formaline induced lipid peroxidation in these animals. AEDM significantly ($p < 0.001$) reduced MDA levels compared to the negative control at 100 mg / kg and 200 mg / kg and at the same time significantly increased SOD ($p < 0, 05$) at all doses of the extract compared to the negative control. AEDM also significantly ($p < 0.001$) increased catalase and glutathione levels. In fact, superoxide dismutase is an enzyme that has the ability to catalyze the superoxide anion into less toxic hydrogen peroxide (Pincemail 2005). Catalase and glutathione have a similar action to catalyze the decrease in hydrogen peroxide (H_2O_2) (Guimard et al. 2007). The increase in these parameters suggests that the aqueous extract of *Diospyros mespiliformis* may have antioxidant power. This effect could be due to the presence in this extract of phenolic compounds such as flavonoids, tannins and alkaloids which have well-known antioxidant powers (Mzid et al. 2017). Histological study of the legs of the animals reveals that formaline caused an alteration in the structure of the dermis and epidermis of the arch of the foot shown in the photomicrograph. Animals treated with the aqueous extract of the plant showed normal structure compared to the negative control. These results suggest that the extract limited the destruction of paw structures in animals. Limiting the destruction of these structures, the extract would certainly have reduced the inflammatory process in the legs of animals and consequently the pain.

Conclusions

The present study proved the properties of the aqueous extract of the bark of the trunk of *Diospyros mespiliformis* on the pain and anxiety of induced arthritis in mice. The aqueous extract of *Diospyros mespiliformis* reduced formaline-induced edema and increased the threshold of nociception in mice. It also improved the state of anxiety in animals. Our plant would therefore have an anti-inflammatory, antioxidant effect and would fight against anxiety

Ethical considerations

The study was approved by ethic Committee of the Faculty of Sciences of the University of Maroua (Ref N°14/0261/Uma/D/FS/VD-RC), according to the guidelines of Cameroonian bioethics committee (Reg N.° FWA-IRB00001954).

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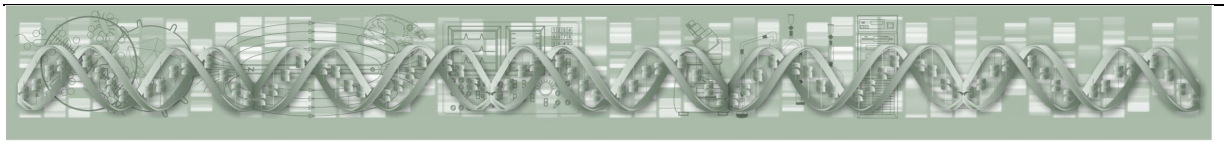

DISTRIBUTION OF THE PRION PROTEIN GENE 23-BP INDEL POLYMORPHISM IN JERSEY CATTLE IN TURKEY

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Abstract

Bovine spongiform encephalopathy (BSE) is a prion disease that is always fatal in cattle and is considered an important risk factor for human health. Genetic polymorphisms that alter prion proteins may be associated with susceptibility or resistance to infectious spongiform encephalopathy. Therefore, we investigated the distribution of the 23 bp indel variant in the prion protein (*PRNP*) gene in Jersey cattle in Turkey. A total of 95 unrelated Jersey cattle (79 of reproductive age and 16 of non-reproductive age) from a private farm in Izmir were included in the study. Genomic DNA was obtained from the milk of reproductive-age cattle and the saliva of non-reproductive-age cattle. A 23-bp indel polymorphism in the *PRNP* gene promoter region was genotyped by polymerase chain reaction (PCR) analysis. The three genotypes of the PRNP 23-bp indel variant were classified as follows: I/I (223 bp), I/D (both 223 and 200 bp fragments), and D/D (200 bp).. The frequencies of the I/I, I/D, and D/D genotypes of the *PRNP* 23-bp indel variant in cattle were 22 (23.16%), 48 (50.53%), and 25 (26.32%). We then examined genotype and allele distribution according to service period. No significant difference was detected in terms of *PRNP* gene 23 bp-indel variant genotype and allele distribution in the groups created according to the service period ($p>0.05$**).** Although the *PRNP* gene 23 bp-indel variant genotype and allele distribution in jersey-type cattle in Turkey did not differ according to service period, our results may benefit the understanding of the genetic characteristics of the *PRNP* gene in cattle breeds.

Keywords: Bovine spongiform encephalopathy, prion protein, variant, PCR

Introduction

Prion diseases, also called *transmissible spongiform encephalopathies* (TSEs), are a group of neurodegenerative diseases that also affect both mammals and humans (Maghsood et al. 2011). This disease primarily targets the central nervous system (CNS) and is presented with a range of neuropathological symptoms. Common TSE pathologies consist of spongy changes, neuronal loss, glial cell activation, and, most importantly, the accumulation of amyloid aggregates (Murdoch et al. 2015). With the accumulation of this abnormal prion protein in tissues, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker syndrome in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in small ruminants, and chronic wasting disease in deer occur (Vaccari et al. 2009). Prion diseases may arise through acquired transmission, in accordance with inherited genetic risk, or through sporadic origins. Acquired prion diseases in cattle are usually transmitted by oral exposure to infectious prions.

Humans can also be transmitted through contaminated human products or surgical instruments. This is known as iatrogenic contagion (Cali et al. 2015).

Prion protein (PRNP), a glycoprotein consisting of four alpha helical chains, belongs to the group of protective proteins, suggesting that it may play an important role in an organism. Being part of the surface membrane indicates that it is involved in signal transmission between cells (Rzewucka-Wójcik et al. 2013,). The bovine *PRNP* gene is localized at q17 on chromosome BTA13. The structure and organization of this gene have been determined (Czarnik et al. 2007). *PRNP* plays a role in infectious BSEs. Resistance to prion diseases in a wide variety of mammalian species is affected by polymorphisms in the *PRNP* gene (Ün et al. 2008). In their study of 7 German cattle breeds, Sander et al. showed that the insertion/deletion (I/D) polymorphism in the promoter region (23 base-pairs (bp) and 12 bp in intron 1 was different between clinically healthy and infected animals (Sander et al. 2004). These indels are thought to affect the binding sites of transcription factors and may affect the expression of this protein (Sander et all, 2005). A 23-bp I/D located in the PRNP promoter contains a binding site for the RP58 repressor protein (Yang et al. 2018).

The Jersey breed has higher efficiency in converting feed into milk. It costs less than other dairy cattle, as they reach a productive age between 2 and 10 months before other dairy cattle (Ahmad et al. 2007).

Therefore, in this study, we aimed to investigate the distribution of a 23-bp indel variant in the Prion protein (*PRNP*) gene in Jersey cattle in Turkey.

Materials and Methods

Study population

A total of 95 unrelated Jersey cattle from a private farm in Izmir, Turkey, were included in the study. Seventy-nine of the cattle were of reproductive age, and 16 of them were not. The cattle were fed on a farm under the same environmental conditions and with the same feed.

Genotyping

Genomic DNA was obtained from the milk of reproductive-age cattle and the saliva of non-reproductive age cows using a commercial kit. Qualitative and quantitative analysis of the isolated DNA was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). *The PRNP* 23-bp indel genotype distribution was determined by the polymerase chain reaction (PCR) method. Forward (5′- GTGCCAGCCATGTAAGTG-3′) and reverse (5′-CCTATTCTGGCTATTGTTGC-3') primers were used for amplification, with initial denaturation at 5 min at 95°C; 2 cycles of 94°C for 30 s, annealing from 68°C to 52° by 2°C decrease for 30 s, respectively; 72°C for 30 s; 30 cycles of 94°C for 30 s, 50° The 25 µL PCR amplification volume contained 50 ng of genomic DNA, 0.5 µmol/L of each primer, 1 × buffer (including 1.5 mmol/L MgCl2), 200 µmol/L dNTPs (dATP, dTTP, dCTP, and dGTP), and 0.625 units of Taq DNA polymerase (Thermo Fisher Scientific, USA). PCR products were identified by electrophoresis on a 2% agarose gel stained with ethidium bromide. To check the results, 10% of randomly selected samples were reevaluated, and a 100% match was found.

STRING analysis

In molecular biology, the STRING database, a biological database and web resource, describes functional interactions between proteins in a cell (https://string-db.org/).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows was used to analyze the data (SPSS Inc., Chicago, IL). The mean and standard deviation were used to show the continuous quantitative variables. The *PRNP* overall genotype distribution was compared with the chi-square (χ^2) test, and the allele and genotype distributions were compared with Fisher's exact test. The p-values smaller than 0.05 were considered statistically significant.

Results

In this study, the PRNP 23-bp indel polymorphism was investigated in 95 cattle populations. Baseline demographic features of the groups are shown in Table 1.

Table 1. Baseline demographic features of the cattle

	n: 95 (%)
Reproductive age	
Yes	79 (83.16)
No	16 (16.8)
Age (years)	
<1	6 (6.32)
1	24 (25.26)
2	7 (07.36)
3	58 (61.05)
Service period (days)	
<90	55 (57.89)
90-180	24 (25.26)
>180	16 (16.84)

The three genotypes of the PRNP 23-bp I/D variant were classified as follows: I/I (223 bp), I/D (both 223 and 200 bp fragments) and D/D(200bp). The frequencies of the I/I, I/D, and D/D genotypes of the *PRNP* 23-bp I/D variant in cattle were 22 (23.16%), 48 (50.53%), and 25 (26.32%). The allele distribution was I allele 92 (48.42%) and D allele 98 (51.58%). The data are presented in Table 2.

Table 2. Genotype distribution and allele frequencies of *PRNP* 23-bp indel variant

***PRNP* 23-bp indel**	**n:95 (%)**
Genotypes	
I/I	22 (23.16)
I/D	48 (50.53)
D/D	25 (26.32).
Alleles	
I	92 (48.42)
D	98 (51.58)

We then examined genotype and allele distribution according to service period. Results are shown in Table 3. No significant difference was detected in terms of *PRNP* gene 23 bp-indel variant genotype and allele distribution in the groups created according to the service period (*p>0.05*).

Table 3. Genotype and allele distribution *PRNP* gene 23bp-indel variant according to service period

	Service period			
***PRNP*-23bp-indel**	**<90 n=55 (%)**	**90-180 day n=24 (%)**	**≥181 n=16 (%)**	**P**
Genotypes				
I/I	11 (20.00)	5 (20.83)	6 (37.50)	>0.05
I/D	30 (54.55)	12 (50.00)	6 (37.50)	
D/D	14 (25.45)	7 (29.17)	4 (25.00)	

Alleles				
I	52 (47.27)	22 (45.83)	18 (56.25)	>0.05
D	58 (52.73)	26 (54.17)	14 (43.75)	

STRING analysis

Analyzing the PRNP protein with the STRING database, we predicted the functional partners of the protein with high confidence and found them as follows: Tubulin alpha-1D (TUBA1), Tubulin beta-1 chain (TUBB1), Tubulin beta-3 chain (TUBB3), Tubulin beta-6 chain (TUBB6), Tubulin beta-5 chain (TUBB), Tubulin beta-4B chain (TUBB4B), Tubulin beta-2B chain (TUBB2B), Tubulin beta-2a chain isoform x1 (TUBB2A), Tubulin beta-4B chain (TUBB4B), and Tubulin beta-4A chain (TUBB4A).

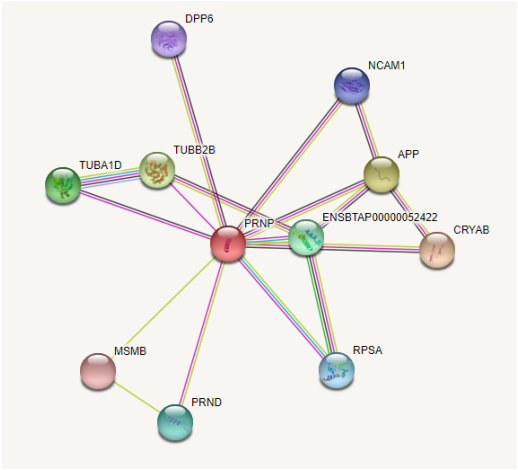

Figure 1. Interactions of Bos taurus PRNP protein, according to STRING database

Discussion

Frequent outbreaks of Prion disease are always fatal and incurable. Therefore, it is a major concern for animal and human health. Classical human prion diseases initially present with memory loss, behavioral changes, and communication problems. Subsequently, the imbalance and ataxia are accompanied by rapidly progressive dementia. Similarly, cattle show progressive neurological and behavioral changes (increased irritability, aggression, and anxiety), altered gait or movement (tremors, weakness, and hind leg ataxia), and weight loss (Murdoch, 2015). Factors controlling interspecific and intraspecific prion transmission are partially understood. Therefore, over the past few decades, there has been much interest and effort in research into understanding prion diseases, their etiology, contagion, and causes.

Prion diseases such as BSE in cattle, scrapie in sheep, and CJD in humans are caused by changes in the PRNP protein. The PRNP protein is a polypeptide that differs slightly between species. Pathogens infecting more than one species can cross species boundaries and affect threatened species, as with prion diseases (Ün et al. 2008). There is no appropriate treatment for BSE in cattle or prion diseases in other mammals. Therefore, it seems appropriate to use genetic selection to eliminate BSE in the cattle population.

The *PRNP* gene consists of three exons and two introns. In exon 3, which is the longest exon, and has an open reading frame (ORF) of 795 bp (Strychalski et al. 2011), one study showed that Japanese black cattle carrying the homozygous del genotype had higher mRNA levels in the medulla oblongata (Msalya et al. 2011). I/D allele frequencies were found to be different in studies conducted with different cattle breeds. The 23del-12del haplotype has been predicted to be associated with an increased risk of BSE. This haplotype is more common in healthy

Holstein-Friesian cattle (Brunelle et al. 2008). The Polish study found a significant association between *PRNP* indel polymorphisms (23 and 12 bp indels) and the susceptibility of Polish Holstein-Friesian cattle to classical BSE. Del variants of both polymorphisms were associated with increased susceptibility, whereas ins variants were found to be protective against BSE (Gurgul et al. 2012). Zhu et al. studied the polymorphism frequencies of two indels (23-bp and 12-bp) in four main cattle breeds (Hereford, Simmental, Black Angus, and Mongolian) from Northern China (Zhu et al. 2011). The del genotype and allele frequency of 23 and 12-bp indels were lower in Mongolian cattle, whereas the ins genotype and allele frequencies were higher than in the other three cattle breeds. In Mongolian cattle, 23-bp ins / 12-bp ins were the main haplotypes, while 23-bp del / 12-bp del were the main haplotypes in Hereford, Simmental, and Black Angus cattle. These results indicated that Mongolian cattle may be more resistant to BSE than the other three cattle breeds due to their relatively low del genotypes and allelic frequencies of 23- and 12-bp indel polymorphisms. The del allele was more common in German cattle, German Holstein, Fleckvieh, Japanese Holstein, and Korean Hanwoo breeds (Ün et al. 2008, Juling et al. 2006, Jeong et al. 2006). Un et al. examined three local cattle breeds of Turkey (South Anatolian Red, East Anatolian Red, and Turkish Grey), del allele frequency was higher in both Anatolian Red breeds, while the frequency of the ins allele (0.62) was higher than the del allele (0.38) in Turkish Gray cattle (Ün et al. 2008). The allele of 23 bp was higher in German Brown, Holstein (Korean), and Braunvieh cattle (Juling et al. 2006, Jeong et al. 2006, Kashkevich et al. 2006). In a study conducted in different races in Turkey, the highest del/del genotype frequency in the promoter region of PRNP was found in East Anatolian Red and Southern Anatolian Red, followed by Turkish Grey. But Anatolian Black and Zavot breeds showed low frequencies.

In the present study, we studied the *PRNP* gene's 23-bp indel variant in Jersey cattle in Turkey. When we examined the *PRNP* 23-bp indel genotype distribution in 95 Jersey cattle, it was the most common I/D genotype (50.53%). Then, we examined the genotype distribution according to the service period. Although the relationship between *PRNP* genotypes and milk yield is not clear, breeding TSE-resistant breeds will not result in a reduction in economically important reproductive traits. There was no significant difference between genotype distribution and service period ($p>0.05$).

Conclusions

Genetic polymorphisms can predict some diseases' susceptibility. We analyzed *PRNP* 23-bp indel variant genotype distribution and allele frequency in Jersey cattle breeds in Turkey. However, species-specific differences must be taken into account when analyzing such data. To further evaluate the association between BSE and this variant, larger sample sizes and studies of different breeds are required.

Author Contributions: All authors have contributed equally to this work.

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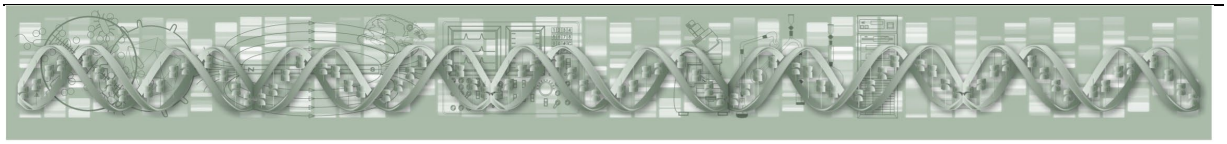

MITOCHONDRIA: AS A PROTAGONIST IN NEUROLOGICAL DISORDERS IN BRIEF OVERVIEW

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Abstract

Neurological disorders pose a great burden in general health. It is not astounding that mitochondrial malfunction emerging as a leading factor in myriad of neurological disorders. Mitochondria are extremely active cell organelles performing various functions, most importantly providing ATP to sustain cellular processes. Mitochondrial dysfunction results in altered neuronal bioenergetics, redox equilibrium and dynamics of cell and acts as focal point of pathogenesis in many human diseases including neurological disorders. Mitochondrial dynamics regulates pathways involving oxidative stress and apoptosis. Often mitochondrial division imbalance and fusion leads to mitochondrial functional impairment. Extreme variations in mitochondrial fusion causes increased mutation rate which along with increased oxidative stress can facilitate development of various neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's diseases and so on. Mitochondria has a key role in regulation of apoptosis. Mitochondrial dysfunction and mutations can have deleterious effects on neuronal functioning as neurons have high energy demand with restricted regenerative potential. Certain neuroprotective agents restores the functions of mitochondria and acts therapeutic regimens of neurodegenerative diseases.

Keywords: Antioxidants; Apoptosis; Mitochondrial DNA; Reactive Oxygen Species; Neuroinflammation; Neurological disorders.

Introduction

Neurological disorders (ND) are the second leading cause of death globally and its prevalence is further expected to increase worldwide. According to the data from 1990 to 2016 it is second leading cause of death next to cardiovascular diseases. Based on the 2015 report, prevalence of spectrum of neurological disorders in India was around 2,394 in 100,000 population. The burden of neurological disorders is expected to rise to 6.77% by 2030. It is anticipated that mortality rate will rise into 12.22% by 2030 (1). Neurons are highly dependent on mitochondria for energy currencies, high energy intermediates and ketone bodies. Mitochondria are essential cell organelles in the cytosol which is the prime source of ATP for neurons, especially in the brain. Mitochondria has its own genome, double stranded circular DNA known as mitochondria DNA (mtDNA). Disruption in the machinery of energy metabolism due to genetic variants can alter the normal homeostasis contributing to the development of neurological disorders, including neurodevelopmental syndromes, neurodegenerative diseases and neuropsychiatric disorders (2). These ND are comprised by a heterogeneous group of diseases and syndromes

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that encompasses different behavioural phenotypes including cognitive and different personality patterns. Specific emotional disturbances, such as autism, Asperger's syndrome, pervasive developmental disorder, attention deficit hyperactivity disorder and bipolar disorder (3).

Mitochondrial stress and morphology might render selectively vulnerable neurons more susceptible to genetic variations, environmental toxins, cellular stress and ageing thereby triggering neurodegeneration (4). Mitochondrial function and properties have important relationships to apoptosis, necrosis, or apoptosis–necrosis hybrids which emerge along a cell death continuum. Mitochondrial mutations mainly affect tissues which requires a large amount of ATP. So, mitochondrial involvement occurs when acute interruptions in O_2 supply to the brain happens. Neuronal energetic defect is seen in cerebral ischemia–reperfusion injury, trauma, toxic exposures and neurodegenerative diseases and causes cognitive/motor dysfunctions (5). Major functions of mitochondria include ATP production through ETC, intracellular Ca^{2+} homeostasis, steroid hormones synthesis and apoptosis during the period of growth (6). Mitochondria are areas of redox reactions which leads to formation of reactive oxygen species (ROS), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) and its intermediates (7).

Neurodegenerative Diseases

Neurodegenerative diseases include Parkinson's disease (PD), Alzheimer's disease (AD), Huntington Disease (HD) and amyotrophic lateral sclerosis (ALS). Most of these diseases revealed the abnormality of mitochondria morphology and impairment of biochemical actions. These variations are often systematic instead of brain limited. Improper functioning of mitochondria may arise from abnormality of mitochondrial DNA and mutant nuclear proteins which interacted with mitochondria either directly or indirectly. Mostly in several cases it is due to decreased respiratory activity and inhibition of specific key regulatory enzymes like pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and cytochrome oxidase.

Superoxide dismutase-1 (SOD1), acts as a universal antioxidant enzyme, it neutralizes the superoxide radicals to hydrogen peroxide, which are further converted to molecular oxygen by other antioxidant enzymes for example glutathione peroxidase and catalase. Predominantly it is localized in cytoplasm, but in ALS affected tissues it was found that both the wild type and mutant SOD1 protein is present in the matrix, intermembrane space and outer membrane of mitochondria. In outer mitochondrial membrane the accumulation and aggregation of mutant SOD1 leads to impairment of mitochondrial protein import and disturbs the mitochondrial function. The oxidative stress was increased in ALS. In post-mortem CNS tissues of ALS, the markers of immune system activation were significantly elevated. Further, in sporadic ALS a peculiar type of mtDNA mutation, called the ≈5 kb common mtDNA or mtDNA-4977 base pair common deletion involving seven protein coding genes and five tRNA genes has an increased frequency (8,9).

The Huntington's disease is caused by triple repeat expansion of CAG in the Huntington (HTT) gene. Generally, the HTT gene interacts with several transcription factors, like p53**,** CREBP-binding protein, Sp1, and PGC1- α (10). Among these, PGC1- α is a transcriptional coactivator that involve in the metabolic pathways of cell and regulation of mitochondrial biogenesis. Despite this, when the mutant HTT gene binds with p53 upregulates the nuclear p53 levels and transcriptional activity, through this it induces mitochondrial membrane depolarization. It is widely considered that 8-hydroxy-2-deoxyguanosine (8-OHdG) levels indicates the oxidative stress and its severity. In AD patients these 8-OHdG levels are increased in cortical brain regions. In AD subjects' brain, the mtDNA with large deletions (including a 4977 base-pair

common deletion) and point mutations are high in hippocampus, parietal gyrus and cerebellum (11).

Mitochondrial DNA

mtDNA contains double stranded DNA ~16.5 kb pairs. It contains 37 genes encoding 13 proteins, 22 tRNAs and 2 rRNAs i.e, 12S and 16S rRNAs. The proteins are required for production of protein subunits of OXPHOS system. It is reported earlier that mitochondrial genome has about 100-fold higher mutation rate as compared to the nuclear genome and the probable reason could be increased mitochondrial ROS causing damage to mtDNA and also less efficient DNA repair mechanisms. This leads to a heterogenous population of mtDNA residing in the same cell and this is called as heteroplasmy. It has been speculated that in postmitotic tissues, mutant mtDNA could be multiplied through clonal amplification. As mitochondria works as powerhouse of cells, mitochondrial mutation produces distinguished phenotypes in tissues requiring high energy like retina, skeletal muscles, myocardium, and brain many syndromes are associated with mitochondrial mutations such as Leigh syndrome, Pearson syndrome and progressive external ophthalmoplegia. Interestingly, in different areas of human brain which is progressing towards aging the mtDNA4977 base pair common deletion has been described (9). There is ongoing research for the use of this deletion as a predictor and prognostic marker in carcinogenesis.

DNA repair in mitochondria

As the mtDNA does not have protective histones they are certainly more vulnerable to ROS attack and hence oxidative stress. Several diseases occurs when DNA repair mechanisms fail and various mutations accumulates. In mitochondria the major repair mechanism is base excision repair (BER) involving precise execution of gap filling and terminal processing (12). Accumulation of mtDNA mutations causes a vicious circle involving oxidative damage, energy depletion and a shoot up in ROS production. In comparison to nuclear DNA, mtDNA endures excessive steady-state damage. DNA damage occurs due to increased levels of ROS and also because of less histone mediated nuclear protection to chromatin. Mitochondria contains active DNA base excision repair proteins, which are all encoded by nuclear DNA. Although they are present at lower levels than in nuclei, these repair proteins play a crucial role in pathogenesis of various mitochondrial diseases (5). Nuclei and mitochondria use variant proteins for base excision repair. 8-oxoguanine DNA glycosylase-1 (OGG1) is a DNA glycosylase enzyme which is encoded by OGG1 gene (13). It is involved in base excision repair. Alternative splicing variants of OGG1exist. The N- terminus contains a mitochondria targeting signal which is required for mitochondrial localization. Also, endonuclease III-like protein (NTH1) is a DNA glycosylase required for the repair of oxidised bases. When DNA glycosylase excises a base, the abasic site is excised by AP endonuclease. Full length APE has a prominent role in mitochondrial repair. DNA ligase IIIβ is located in both nucleus and mitochondria and it functions as DNA ligase in mitochondria base excision repair. Isoform 1 is targeted to mitochondria (14). DNA polymerase γ (POLG) is consideeed to be totally responsible for mtDNA base excision repair and replication. Human POLG is a nuclear-encoded gene product and is required for mitochondrial BER (5).

Role of mitochondria in neurological disorders

It has been speculated that a significant number of syndromes arises with marked neurological phenomena in the spectrum of mitochondrial disorders. But there exists a variability in the

clinical manifestation of mitochondrial function disruption, provided that there exists a limit in the degree of mitochondrial deficiency for the clinical expression of the disease and for their phenotypic effects later on. Thus, the pathophysiology and clinical manifestation are aggravated in a chronic and continuous manner, in most of the diagnosed cases of mitochondrial dysfunctions, along with the increasing age of the subjects. Hence, it is quite acceptable that organs which have very high rate of energy demand would be more sternly affected by the mitochondrial dysfunction than others which have a comparatively low level of energy requirements. So, it is the brain, the skeletal muscles, and the heart which have a particular type of involvement in adolescence and adult population, though multi-system manifestation of mitochondrial dysfunction is also a common phenomenon, especially seen in childhood (15).
Therefore, a finer look into mitochondrial genes and molecular basis of mitochondrial biology can help in better perception of neurological disorders (5,16). Regarding this, a differing intensity of mitochondrial dysfunction and intrinsic mitochondrial mediated cell death mechanisms might be important factors in the pronouncement of diseases which usually ranges along a sequence of apoptosis–necrosis cell death. With therapies attacking specific mitochondrial properties, pathways, or molecules, like mitochondrial permeability transition pore (mPTP), might be noteworthy for evolving newer mechanism-based pharmacotherapies for a whole spectrum of neurological disorders. An example is restless legs syndrome, a neurological sensory disorder caused mainly due to deficit in the levels of mitochondrial ferritin (5).

Mitochondrial stress and immunity

A mitochondrial biogenesis deficit in neuronal cells was found to be functionally linked with the clinical advancement of neurological disorders. The endosymbiotic theory explains that mitochondria is evolutionarily derived from alpha-proteobacteria. It was found that mtDNA possess significant structural similarity with bacterial DNA. Therefore, mtDNA fragments boost host innate immunity and other acquired factors along with inflammation by activating cytokine storm and other inflammatory markers. The etiological factors of different neurological disorders are even though different, the activation of immune system by fragmented mtDNA takes place in a common biochemical pathway (17). In this way, circulating cell free mtDNA produces significant and heterogenous inflammatory responses comprising broad spectrum antimicrobial immunity and neuro-immunological disorders which proves its gravity in neurological disease progression (18).

Biomarkers of mitochondrial mediated neurological disorders

Mitochondrial miRNAs in neuroinflammation
miRNAs (micro RNA) are small non coding RNA which inhibit or degrade endogenous mRNA transcript. Mitochondrial miRNAs, found within mitochondria, are commonly known as mitomiRs. miRNAs control various functions of mitochondria like OXPHOS by miR-338 and COXIV, cell signalling is controlled through miR-696 and PGC1α, fission and mitophagy is managed via miR-30, p53/Drp1 and miR-21, PTEN respectively. Some notorious mitomiRs include miR-155, miR-181c, miR142-3p/5p and miR146a and they might have a double origin. They can be either nucleus derived cytosolic precursor form which gets processed inside mitochondria or could originate from the mitochondria directly (19,20). Disruption in miRNA function occurs most probably by oxidative stress. As the miRNA moves inside the mitochondria, the miRNA dyregulations taking place either in the nucleus or cytosol might get translated into mtDNA alterations and alters mtDNA transcription. Due to blunting of the respiratory complex like cytochrome oxidase I and III by massive increase in ROS compounded

by miR-13a and miR-181c, mtDNA fragmentation occurs (17). Mitochondrial function can also be affected by the action of mitomiRs on mtDNA. In fact, in a wide assortment of genes the genetic expression is modulated by the mitochondria as a main messenger while they deliver miRNAs towards the intracellular compartments. In the cytosol mitophagy is subdued because of abnormal spreading of miRNAs. Thus, damaged mitochondria, mtDNA and miRNAs exit from the cells is disrupted (20).
miRNAs such as miR-155, miR-146a and let-7b are found to be involved in neuroinflammation. They are found circulating in extracellular fluids inside the exosomes and they behave similar to Damage-associated molecular patterns (DAMPs) for activating Toll-like receptor (TLR7) in a manner similar to Circulating cell-free mitochondrial DNA (ccf-mtDNA) (21). They are found circulating in extracellular fluids inside the exosomes. These DAMP-like miRNAs are present in mitochondria. Also, their activity is triggered by NF-kB, which is facilitated by mtDNA fragments (22). These findings show similarities between mtDNA and miRNAs as potential biomarkers for neuroinflammatory disorders.

Circulating cell-free mitochondrial DNA
ccf-mtDNA are short sections of mitochondrial DNA which are released by cells undergoing stress or any other pathology. They are recognised by immune system and activate inflammatory reactions (23). It has been proven as a diagnostic and prognostic biomarker as it could be used to detect the degree of damage in several diseases like malignancy, trauma, various microbial infections, cardiovascular accident and myocardial infarction (24). It is stable in extracellular fluids in plasma as well as the Cerebrospinal fluid (CSF). mtDNA possess higher resistance to nuclease-dependent degradation when compared with the nuclear DNA. This shows mtDNA as a highly advantageous stable biomarker (25).

ccf- mtDNA in neurological disorders
As the mitochondrial DNA particles are liberated from the cell through the cell membrane, they manifest themselves as ccf-mtDNA in the extracellular space (26). mtDNA particles act as danger-associated molecular patterns (DAMPs) to activate host innate immunity and augment inflammatory response similar to pathogen-associated molecular patterns (PAMPs). This mechanism takes a turmoil due to binding to Toll-like receptor 9 (TLR9) and successive triggering of the stimulator of interferon genes (STING) pathway. Therefore, DAMPs accumulation activates macrophages which are residing in the cells and stimulates tissue infiltration done by the leukocytes. When such a molecular mechanism takes place between DAMPs and PAMPs it might also progress to non-differentiable clinical responses which are later accompanied by infective and non-infective damage. In this context, for the clinical diagnosis the detection of genetic profile of associated pathogen remains quite essential, and quantification of ccf-mtDNA is coming up nowadays as a novel biomarker for disease screening and prognosis at early stages (27). In the early phase of infection, when mitochondrial and cell membrane damage is less, the spread of ccf-mtDNA functions as an attempt to provoke immunity against the microbes, removes non- functional mtDNA fragments and thereby preserves mitochondrial function (28).

Mitochondrial DNA variants and its associated neurological disorders

mtDNA possess higher mutational rate compared to nuclear DNA, associated with single nucleotide polymorphisms (~79%), deletions (~15%), copy number variations (~3%), insertions (~2%) and other genetic rearrangements (1%). Mostly these variants influence mitochondrial proteins and the rest of them is associated with tRNA and rRNA. Due to poor fidelity of DNA polymerase in mitochondria, ROS levels imbalance occurs and this drives

aging process. ROS promotes oxidative stress, protein damage, mitochondrial dysfunction which can contribute to ND. In mitochondria, the OXPHOS mechanism gets interrupted when ETC genes mutation occurs. This condition diminishes ATP production and causes epilepsy and LHON (Leber's hereditary optic neuropathy) syndrome. Variations of genes of neuronal synapses grounds structural and functional alterations, this favours autism spectrum disorder (ASD), attention deficit hyperactivity disorder. In LHON the genes involved in mitochondrial replication, transcription or translation were mutated. Alzheimer's disease is the first most common ND affected due to amyloid-β aggregates. Mitochondria, an important controller of apoptosis are affected by amyloid-β aggregates. Misfolded amyloid-β protein aggregates have significant effect on components of the electron transport chain (29).
Mutations of genes like PINK1 (PTEN-induced kinase 1) and Parkin (E3 ubiquitin protein ligase) indicates loss of mitochondrial quality control, altered calcium homeostasis and reduced OXPHOS. PINK1 and Parkin acts as potential regulators of 'mitophagy' for defective mitochondria. Mutations of downstream proteins in mitophagy (NIPSNAP1 & 2) attributes to parkinsonian phenotype (30). In HD, a molecular cause of this neurodegenerative disease is trinucleotide repeats of CAG. This corresponds to polyglutamine tract of HTT protein to form misfolded aggregates. There is strong association between aging and mtDNA variations which progresses MELAS (Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), LHON and PD (31). In MELAS and MERRF (myoclonic epilepsy and ragged-red fibres), it was reported that mutation of mitochondrial tRNA takes place which alters its structure and functions (32). Variants in mtDNA causes cognitive impairment, developmental delay, slow learning capability and behavioural abnormality. In ASD, HD, BD and Leigh Syndrome besides nuclear genes, mtDNA defects were also seen in hereditary pattern. So far various studies reported that, 105 out of 158 mtDNA mutations, 13 mitochondrial proteins coding genes had functional involvement of coding region with mtDNA variants (33).

Single nucleotide polymorphisms of mtDNA

Single nucleotide polymorphisms (SNPs) are the simplest DNA variations among the individuals. Out of 152 SNPs associated with various neurological disorders, 4 were related to non-coding mtDNA region and 3 were related to D-loop locus. In bipolar disorders variants are found in D-Loop region, MT-ND1, MT-ND2 and MT-CYB protein coding genes. Individuals suffering from psychiatric disorders like schizophrenia and bipolar disorders also have mtDNA mutations. Genetic variation in promoter region of NDUFV2, a mitochondrial complex 1 gene is associated with bipolar disorder. Many non-synonymous mutations were found to have affected the mitochondrial genes like MT-ND6, MT-ATP6, MT-CYB and MT-ND2 in schizophrenia. mtDNA screening was done, it was speculated that NARP (neuropathy, ataxia, retinitis pigmentosa), dementia, RTT, learning difficulty, ID present a comparatively decreased amount of mutations. NARP has been associated with 4 mutations at two sites in the ATP6 gene(34). mtDNA polymorphisms like A10398G, T3644TC, T16519C and T12027C, T195C are associated with SCZ and BD respectively. Few neurological disorders like ASD, Leigh Syndrome, MELAS and myoclonic epilepsy with ragged red fibres (MERRF) were also reported to have increased mtDNA mutations. In ASD mutations are located in MT-CO1 coding for Cytochrome C Oxidase 1 and second mutation in MT-CO2 coding for Cytochrome C Oxidase 2. These genes are involved in ATP production and reducing oxidative stress. In Leigh Syndrome point mutations were causative agent in seven out of 13 mitochondrial protein coding genes. However, maximum mutations exist in MT-ATP6, MT-ND3, MT-ND5(35). MT-TL1 was also linked with seizures, ASD and Leigh Syndrome. MERRF is a disorder affecting mainly muscles and nervous system. Usually, symptoms appear during childhood or adolescence. Mutation in MT-TK is the chief cause of MERRF with most of the mutation

confining to tRNA (36). Various mtDNA mutations, alterations associated with copy number variations, insertions and deletions interfere in the exact functional pathways of the central nervous system causing various neurological disorders. Furthermore, mtDNA deletions usually lies between the *MT-TT* and the *MT-TC* transcripts, whereas the locus between the two transcripts *MT-TP* and *MT-TN*, has no deletions. Different types of mutations were identified with protein coding genes and also with spliced RNA transcripts but surprisingly the tRNA molecules *MT-TA* and *MT-TN* were not associated with any type of mutation (37).

Mitochondrial mediated apoptosis in Neurological disorders

"Mitophagy" is a programmed mitochondrial removal mechanism which regulates mitochondrial quality as well as quantity. Impaired mitochondria is severe threat for neuronal function because lack of ATP generation and excessive production of ROS. Increased ROS and oxidative stress causes mitochondrial membrane depolarization i.e, loss of matrix metalloproteinases (MMPs). mPTP switches on PINK1/Parkin-mediated mitophagy pathway. PINK1/Parkin pathway mainly associated with Parkinson's disease. Cell death is induced by increased free radicles, oxidative stress, deficit of neurotrophic factors and multiple other factors impairing mitochondrial function. Organelle of endomembrane system like endoplasmic reticulum (ER), chiefly balances intracellular Ca^{2+} levels along with mitochondria. The Ca^{2+} homeostasis in the ER and mitochondria are regulated by the Bcl-2 family proteins and are thus the vital components responsible for apoptosis. These changes modulate mitochondrial permeability and promotes release of proapoptotic factors such as Bax and cytochrome C to activate caspases. Finally, released caspases promotes internucleosomal cleavage of DNA in the cells (38).

Protagonist of mitochondrial antioxidant defense system against oxidative stress in ND

Mitochondria generates endogenous ROS as by-products of redox reactions. ROS are superoxide radical, hydroxyl radical and hydrogen peroxide which damage organellar macromolecules including mitochondrial proteins, enzymes, ETC complexes and iron–sulphur clusters. Variations in intra- and intermitochondrial redox environment leads to release of free radicals. The formation and release of ROS was processed in the form of regenerative cycle which was termed as ROS-induced ROS release (RIRR). At high levels of ROS favours oxidative stress, this terminates formation of mitochondrial permeability transition pore (mPTP) and then destruction of mitochondria. It propagates from mitochondrion to mitochondrion, of the cell itself. Increased oxidative stress also promotes lipid peroxidation which in turn releases malondialdehyde in several neurological disorders. The mitochondrial matrix contains several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and thioredoxin reductase (TrxR). These enzymatic antioxidants quench the reactive oxygen species and detoxified into non-toxic products as shown in Figure 1.

Several metals for example copper, manganese and zinc acts as cofactors for some of these enzymes. Non-enzymatic antioxidants such as Vitamin A, C, E, glutathione, glutaredoxin, thioredoxin and peroxiredoxin (PRx) are protects mitochondria from ROS and RNS such as superoxide radical, hydroxyl radical and hydrogen peroxide by quenching mechanisms (39).

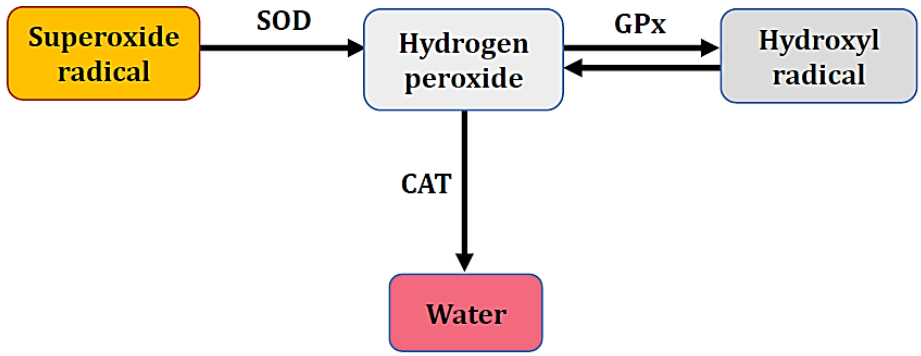

Figure 1. Detoxification of reactive oxygen species by enzymatic antioxidants.

Protection of mitochondria by neuroprotective agents

In neurological disorders, various molecules have been investigated which enhance bioenergetics homeostasis mechanism in the mitochondria. For testing their efficiency in these disorders, creatine and CoQ_{10} are in phase III of clinical trials for AD, HD and PD (40). Methylene blue and photomodulation are used on animal models to increase the energy production and to reduce oxidative stress and neuroinflammation. Several neuroprotective agents like lipoic acid, creatine, CoQ_{10}, nicotinamide, riboflavin are also targeted for their beneficial effect on mitochondrial functions which is shown in Figure 2.

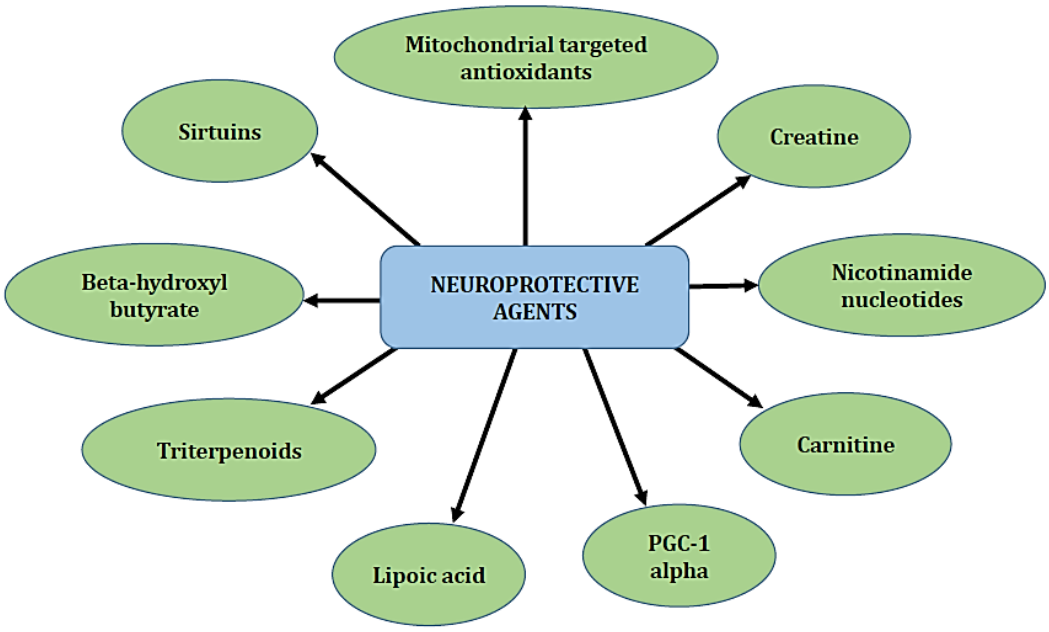

Figure 2. Restoration of mitochondrial function by several neuroprotective agents.

Creatine

Creatine is an organic compound which is found naturally in muscle cells. It is nitrogenous guanidine and it provides energy to all cells throughout the body, especially muscle and nerve cells. It is transported into the brain and skeletal muscle by a sodium-dependent creatine transporter. Mitochondrial creatine kinase catalyses the reversible reaction forming phosphocreatine and it is the main source of high energy phosphates. This system is vital for maintaining energy homeostasis in the brain. A high ATP/ADP ratio is of prime importance for ATP supply at sub-cellular level, to adjust high ATP free energy. This mechanism also minimizes the loss of adenosine nucleotides, thereby preventing cellular dysfunction. In

addition, it was found that glutamate-treated neuronal/glial cells had more viability when Ras/NF-kappaB signalling was inflected with creatine (41) . This modulation augments differentiation of cultured GABA-IR neurons. Creatine also facilitates a significant level of neuroprotection against glucose, serum deprivation and 3-nitropropionic acid (3-NP) induced toxicity (42).
NMDA110 produces striatal excitotoxic lesions which could be efficiently reduced with the oral administration of 1% creatine in the diet. Malonate-induced striatal lesions could be controlled with the use of creatine along with nicotinamide. It gives refinement in cognitive and motor functions, brain atrophy decreases, brain ATP production is enhanced. Also, striatal neurons atrophy and Htt-positive aggregates formation is delayed. Glutamate and β-amyloid toxicity is prevented with the successive usage of creatine. Creatine is used in clinical trails for Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis after its beneficial effects in experimental studies in animals. Creatine monohydrate, CoQ10 and lipoic acid combination was used in a randomized, double-blind, placebo-controlled trail and it was shown to have protective effect in neurological disorders. Recommended dose of creatine is 8 g/day for 16 weeks by oral route. This dosage is safe, endurable and has good bioavailability to the brain. Also, creatine reduces serum 8-hydroxy-2-deoxyguanosine levels which is a novel biomarker of increased oxidative stress and neuroinflammation (43).

CoQ_{10}
In order to accept electrons from complex I and II in the ETC, a biological cofactor CoQ10 (ubiquinone) plays an essential role, as an uncoupler of mitochondrial proteins. CoQ10 interacts with α-tocopherol, to directly scavenge free radicals, in mitochondrial inner membrane, hence it also serves as an anti-oxidant. CoQ10 also acts as an anti-apoptotic factor, by inhibiting Bax mediated mitochondrial apoptotic pathway, and downregulating mitochondrial permeability transition (MPT). Additionally, CoQ10 can also activate uncoupler mptpproteins (UCP) exert neuroprotective effects, associated with marked neuroprotection against the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity through reduction in mitochondrial-free radical generation (44). Further, in experimental models of stroke and epilepsy an increased expression of the mitochondrial UCP was found to have protective role against neuronal damage. CoQ10 has also been reported in protection against iron-induced apoptosis in dopaminergic neurons, as it destabilizes preformed beta-amyloid fibrils and exerts anti amyloidogenic effect. Further, inhibits the MPT pore and protects CoQ10 SHSY5Y neuronal cells from β-amyloid toxicity. Similarly, mitochondrial membrane potential can be maintained by pre-treatment of neuronal cells with CoQ10 during oxidative stress which reduces the ROS generation by mitochondria (45).

Mitochondrial targeted antioxidants/peptides
Mitoquinone (MitoQ) is a potential mitochondrial targeted antioxidant, increasing enzymatic antioxidant activity, including SOD and GPx, whereas it decreases the levels of malondialdehyde (MDA). MitoQ is a derivative of CoQ10, it blocks the mitochondrial ROS overproduction and lipid peroxidation. It attenuates the deficit in motor functions and loss of oligodendrocytes. In ICH, demyelination and axon swelling is reduced thereby decreasing cell death (46). Elamipretide (SS-31) is a novel mitochondrion targeted antioxidant. It was found to modulate SIRT1 levels, reduce oxidative stress, ROS and inflammation. Also, it ameliorates mitochondrial membrane potential and glutathione content (47). MitoQ augmented the Nrf2 nuclear translocation and also upregulates the expression of Nrf2 downstream proteins, such as quinone oxidoreductase-1 and heme oxygenase-1 (48).

Triterpenoids

Triterpenoids are diverse group of natural products. Structurally they are cyclic compounds having a carbon skeleton which are derived from squalene (C30 hydrocarbon) by cyclization. Usually, 5 carbon isoprene units are linked to it in various positions and they are either aldehyde, carboxylic acids or alcohols. Many of them have anti-inflammatory and anti-carcinogenic properties. To prevent the brain from neuroinflammation and oxidative stress, some triterpenoids are being used like Celastrol, lupeol, oleanolic acid, ursolic acid, betulinic acid, pomolic acid, uvaol, asiatic acid and tormentic acid (49). For preventing the progression of neurodegenerative diseases like AD, PD, HD and ALS, few experimental studies have reported that these compounds have a potential role and thus could be used as a treatment (50). Synthetic triterpenoids are also used as they have antioxidant and antinflammatory properties. CDDO-MA is a synthetic triterpenoid prepared to induce signalling cascade of Nrf2/ARE (antioxidant response element). Once Nrf2 is modulated by triterpenoid, it dissociates from Keap1. Next step is translocation to nucleus, and followed by binding to the ARE promoter sequences which causes activation of cytoprotective genes involved in reducing oxidative stress and neuroinflammation. Interestingly, triterpenoids have good anticancer efficacy. Therefore, they are utilized as chemo preventive agents for tumours like leukemia, multiple myeloma, osteosarcoma, breast and lung cancer (51).

Nicotinamide nucleotides

Nicotinamide nucleotides such as NAD+/NMN are hydrophilic amides that maintain the neuronal membrane integrity and protects neurons from apoptosis. NAD+ is synthesised from nicotinamide, with an intermediate nicotinamide mononucleotide (NMN) by the catalysis of an enzyme nicotinamide phosphoribosyl transferase (NAMPT). Even though neurons have low levels of NAMPT, the requirement of NAD+ is essential for normal functioning. NAD+ is mainly associated with energy yielding metabolic pathways and maintains normal homeostasis of neurons. NAD+ is an essential cofactor maintaining mitochondrial fitness and development of neurons from its precursors. Depletion of NAD+ leads to neuroinflammation, synaptic dysfunction and neuronal degeneration in Alzheimer's disease, Parkinson's disease and retinal degenerative diseases (52).

mtDNA are predisposed to various exogenous physical and chemical DNA damaging agents, thus increases the risk of neurodegenerative diseases. Accumulation of mutations and DNA damage hastens aging process and its associated diseases. Poly(ADP-ribose) polymerases (PARPs) are activated in response to DNA damage through reactive oxygen species. NAD+ acts as substrate for three important classes of enzymes like sirtuins, PARPs and cyclic ADP-ribose (cADPR) synthases, thereby protecting the cells from oxidative stress and apoptosis (53). NAD+ prevents neurons from ischemic brain damage and rejuvenates the remyelination process. Nicotinamide riboside improves the cognitive function and hippocampal synaptic plasticity in mice models. Nicotinamide precursors like nicotinamide mononucleotide (NMN), the immediate precursor to NAD^+ by a single step process with an enzyme NMN adenyltransferase (NMNAT) improves bioenergetics in cells and ameliorates disease phenotypes (54). In retinal degenerative diseases such as glaucoma and Leber congenital amaurosis, NAD^+ metabolism is disrupted. Hence, NAD supplementation is considered a novel therapeutic target in these conditions. Additionally, NMN reduces lactic acidosis and serum IL-6 levels which are strong predictive markers of mortality in metabolic deranged condition such as haemorrhagic shock (55). So, NMN is a key precursor for therapeutic implications to increase NAD^+ levels (56). Nicotinamide prevents MPTP induced neuronal degeneration. Wallerian degeneration, commonly occurs in chronic degenerative diseases and nicotinamide

is found to be preventive in neuronal degeneration (57). Therefore NAD+ could be used for the treatment of neurodegenerative disease, ischaemic injuries and trauma.

Lipoic acid

Lipoic acid is an antioxidant available as a dietary supplement. It is present in low amount in food. Recently it has been considered as an effective aid in neurological disorders due to its modulating effect in signal transduction and gene transcription. It contains a disulphide bond and is an essential cofactor for oxidative decarboxylation of α-keto acids like pyruvate dehydrogenase (58). For the progression of neurodegenerative disorder, it has been reported that aging is the major risk factor. Factors associated with rapid ageing are oxidative stress, disruption in bioenergetic machinery causing cognitive and motor function decline. As lipoic acid functions were explored through in vivo and in vitro experimental studies it showed protection against oxidative stress, anti-apoptotic properties, and anti-inflammatory actions in neurodegenerative disorders (59). Most importantly, lipoic acid augments the epigenetic modification of the Nrf-2 required for supporting structural integrity and enabling mitochondrial activity in a proper way (60).

Carnitine

L-carnitine is an amino acid, found in the body (also available as a dietary supplement). It produces energy during beta oxidation as it transports fatty acids from the cytoplasm to mitochondria. It could be converted to other forms such as acetyl-L-carnitine (ALC) and propionyl-L-carnitine. Due to the antioxidant and neuromodulatory properties of acetyl group donated by ALC, they are considered as potential targets for treatment in neurodegenerative diseases. It also has a protective action against MPTP toxicity. Dietary supplementation of ALC is given because of its analgesic effects in neuropathies (61). Neuroprotective and anti-apoptotic action of ALC have also been investigated and could be used in future.

β-hydroxybutyrate

Ketone bodies are used as a treatment modality for various neurological disorders due to their protective effect on neurons. Ketone bodies undergo oxidation especially when glucose supply is low in brain as most of the neurons do not effectively generate high-energy phosphates from fatty acids during starvation. β-hydroxy butyrate, a ketone body, also has neuroprotective action with distinct role in MPTP toxicity (62).

PPARG (Peroxisome proliferator-activated receptor-gamma) coactivator 1 alpha

PPARG coactivator-1 alpha (PGC-1α) is a transcription coactivator that regulates cell metabolism and mitochondrial biogenesis. Along with cell metabolism PGC-1α also emerged as an important factor in the induction of many antioxidant processes by enhancing the expression of several transcription factors like nuclear respiratory factors (Nrf-1 and Nrf`-2). It is also found to be involved in regulating the expression of mtDNA transcription via mitochondrial transcription factor A (TFAM) which is coactivated by Nrf-1. Nrf-1, together with Nrf-2, mediates the genomic coordination between nuclear and mitochondrial genomes by directly regulating the expression of several nuclear-encoded ETC proteins. Nrf-2 is also an emerging regulator of cellular resistance by controlling the basal expression of an array of antioxidant response element–dependent genes, which triggers up-regulation of antioxidants (63).

The diverse role of PGC-1α reduces oxidative stress by inducing the expression of antioxidant enzyme systems. So, overexpression of PGC-1α could completely rescue mitochondrial biogenesis and mitochondrial deficits through inhibition of 5'-adenosine monophosphate-activated protein kinase (AMPK) activity (64). The same has been confirmed in animal studies

where the dopaminergic neurons in PGC-1α null mice are much more susceptible to neurological disorders which include parkinsonian-like features. Conversely, PGC-1α overexpression protects neural cells from oxidative stress-induced by the mitochondrial toxin, MPTP (65). Therefore, PGC1α is also a powerful controller of mitochondrial metabolism and rescues mitochondrial homeostasis.

Sirtuins

Sirtuins or SIRTS belong to the family of NAD-dependent histone deacetylase and play a major role in cell functioning by regulating cell metabolism and cell survival. Activation of the sirtuin helps in the extension of cell longevity and delay the onset of age-related neurodegenerative disorders (66). The same has been confirmed in the mouse model, where the activation of SIRT 1 by resveratrol increased the survival of motor neurons in ALS mice and also decreased neurodegeneration in AD mice. This mechanism works through decreasing the acetylation of SIRT1 substrates such as PGC-1α and p53 (67).

Conclusion

In conclusion, genetic and environmental risk factors are key factors in maintaining mitochondrial function, kinetics and mitophagy during the pathogenesis of neurological disorders. Attenuation of free radicals and oxidative stress could be done by enhancing expression of protective genes such as Nrf-2, enzymatic antioxidants, PPARG coactivator-1 alpha, sirtuins and also suppression of mitochondrial apoptotic mechanism by inhibiting Bax translocation and cytochrome C release from the mitochondria. Furthermore, MitoQ administration leads to activation of the Nrf2-ARE pathway. Overall, neuroprotective agents normalize the redox balance and maintains mitochondrial homeostasis. This indicates mitochondrial approaches of various components exhibit neuroprotective effects and has therapeutic potential in prevention and delaying the progress of neurological diseases. Finally, continuous progress is going on to explore the basic mechanism underlying the mitochondrial functional pathways, it is widely believed that antioxidant therapy in neurological disorders is most likely to precede breakthrough in the near future.

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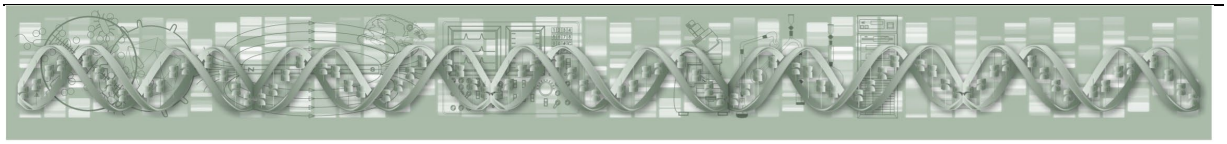

THE MOLECULAR BASIS OF THE FRAGILE X SYNDROME

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Abstract

This analysis aimed to clarify the molecular basis of fragile X syndrome and explain the role of genetic material in the genetic disease's development and treatment. Fragile X syndrome is an X-linked mutation inheritance disorder. The mutated gene is called FMR-1. This is important for normal brain development and synaptic plasticity, which was verified and recognized in 1991, and this has become a hope for more clarification. FMR1 influences the translation of messenger RNA (mRNA), but identifying functional targets was complex and directly related to translational control and showed that dysregulated translation initiation signalling was observed for the FMR1 gene in the FMR1 knockout mouse model of FXS.

Because of the epigenetic alteration, such as hypermethylated at the DNA promoter region, and chromatin modification, such as H3K9 methylation, the FMR1 gene can be imprinted. Still, their mechanisms of aberrant epigenetic marks play a role in the etiology of many neurodevelopmental disorders, some of which we still do not fully understand and need to show more. The opportunities for epigenetic markers to map and alter epigenetic marks and the potential for therapies based on epigenetics and noncoding manipulation. For neurodevelopmental and behavioral conditions, including mental retardation, autism, anxiety, and mood disturbance, FMR1 loss of control is a model.

Most studies have focused on the new and effective approach for Fragile X syndrome, which is Gene therapy is unarguably the definitive way to treat and possibly cure genetic diseases. Many of them are under clinical trial, but more studies, such as the CRISPR/Cas9-based method, should be approved. Adeno-associated viral (AAV) vectors are highly effective for generating models. Most research is used in the mouse model of fragile X syndrome, where AAVs have been used to express fragile X mental retardation protein (FMRP), which is missing or highly reduced in the disorder. The vast expansions need southern blotting in myotonic dystrophy. Fragile X is diagnosed by a form of Southern blotting that relies on the size and the FRAXA gene's methylation status. Almost always, genetic testing is performed by PCR. The few Southern blotting uses include checking for significant destructive gene rearrangements and complete mutations of fragile X and myotonic dystrophy. Expansions suppress the expression of closely adjacent genes, causing loss of function. A named FMRI (fragile-X mental retardation syndrome) gene cDNA probe. Some unique molecular mechanisms, such as CGG expansion in Fragile-X syndrome, can make a particular sequence change in a gene far more probable than any other change.

Keywords: X-linked intellectual, FMR-1 triplet expansion region, RNA-binding protein, X mutation, DNA methylation, and Gene therapy

Introduction

Fragile X syndrome (FXS) is a genetic disorder believed to raise the likelihood of cognitive dysfunction and socio-emotional difficulties (Bartholomay et al. 2019). The mental, behavioral,

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and physical phenotypes differ by sex, with the X-linked inheritance of the mutation affecting males more strongly (Crawford et al. 2001). Owing to the full mutation, the fragile X mutation that segregates in a family is most frequently detected by a child with fragile X syndrome, with symptoms such as developmental delay or mental retardation (Sherman et al. 2005). In 1969, in 3 generations of a family, Lubs first identified a separate fragile site on the X chromosome segregating with intellectual disability. In 1991, the association of the Xq27.3 fragile site with X-linked intellectual disability was confirmed (Ciaccio et al. 2017).

For many medical conditions related to FXS, including epilepsy, and chronic otitis media (O.M.), patients with FXS can be seen in clinical settings (Kidd et al. 2014). Hyperactivity is the most common behavioral concern in children with FXS. In approximately 80 percent of people with FXS, attention deficit hyperactivity disorder (ADHD) is diagnosed (Cowley et al. 2016). Significant progress has been made in both the clinical aspects of the condition and its mechanistic basis; hence, when offering anticipatory guidance, primary care physicians must be familiar with these developments (Visootsak et al. 2005).

The animal model study has resulted in the development of possible novel pharmacological therapies that address the fragile X syndrome's underlying molecular defect rather than the resulting symptoms (Hagerman et al. 2014). Although there is evidence of physical symptoms, mostly premature menopause, and mild external features of the fragile X syndrome among premutation carriers, premutation is associated with no effects (Mazzocco 2000). As highlighted by metabotropic glutamate receptor antagonists and gamma-Aminobutyric acid receptor modulators, knowledge of its pathophysiology has led to the creation of several targeted FXS therapies (Sutherland et al. 1991).

Prevalence of Fragile X syndrome

Soy is ingested in different ways by many adults and children, but little knowledge is available on possible neurological side effects. Prior work suggests a correlation in mouse models of neurological disease and children with autism between the ingestion of soy-based diets and seizure prevalence (Westmark et al. 2020). Over the past ten years, rapid advances in the genomic medicine world, including introducing whole-genome sequencing into state-funded/subsidized healthcare systems, such as the NHS (National Health Service), now suggest extending population-level genomic screening services is at least technologically feasible (Boardman 2020). The most common hereditary cause of mental retardation is Fragile X syndrome (FXS, OMIM 300624), affecting 1:3000–1:4000 males and 1:6000–1:8000 females (Hantash et al. 2011).

Around one in 2,500 is the frequency of the complete mutation allele (Cordeiro et al. 2011). Therefore, it is estimated that 37,000 males (1 in 3,847) and 38,400 females (1 in 3,847) bear the fragile complete X mutation within the U.S. population (Kronk et al. 2010). However, data on the relative prevalence, frequency, and severity of problem behaviors shown by FXS boys are minimal compared to mixed-etiology I.D. boys who also demonstrate problem behaviors (Hall et al. 2016). Therefore, there will be inadequate fragile X mental retardation protein (FMRP) in the affected person. Lower repeat numbers are referred to as "premutation", ranging from 55 to 200, whereas those between 45 and 54 are referred to as "intermediate", and those between 35 and 44 are referred to as "in the high normal range" (De et al. 2014).

Molecular basis of Fragile X syndrome

Fragile X mental retardation gene (FMR1)-coded protein reduction causes fragile X syndrome. This genetic disorder causes several developmental issues, including learning disabilities, cognitive impairment, and behavioral disorders (Lyons et al. 2015). Currently, clinical

participation in males and females bearing the FMR1 premutation is a real health issue (Mila et al. 2018). Many of the common fragile sites, such as those induced by APH, a DNA polymerase alpha inhibitor, are distributed with a heterogeneous composition across megabases of DNA. These regions are also enriched with high versatility and low stability for A + T-rich sequences and sequences (Yudkin et al. 2014).

In 8 of four cosmids contiguous YAC DNA, a gene (FMR-1) was identified that expresses a 4.8 kb message in the human brain (Verkerk et al. 1991). The Fragile X Mental Retardation 1 (FMR1) gene encoding the FMR1 protein is essential (Yrigollen et al. 2012). Most cases of FXS result from the expansion of a CGG·CCG repeat in the 5′ UTR of the FMR1 gene that leads to gene silencing (Kumari and Usdin 2010). Patients with FXS have more than 200 CGG trinucleotide repeats. On the other hand, although premutation carriers (55 to 200 repeats) are not affected by the classic FXS phenotype, they can have other medical, psychiatric, and neurological problems (Saldarriaga et al. 2014). The lengthening of the CGG repeat, the cause of FXS, is hypothesized to occur with the addition of length-specific interruptions (e.g., AGG, CGA, or CGGG) at the distal end of the CGG array with incremental additions of smaller CGG arrays (Greenblatt et al. 2018).

Mutations cause the most common inherited human autism spectrum disorder in the Fragile X mental retardation 1 gene (FMR1). FMR1 affects the translation of messenger RNA (mRNA), but it has not been easy to establish realistic targets (Bardoni and Abekhoukh 2014). The FMR1 encoded protein, Fragile X mental retardation protein (FMRP), is an RNA-binding protein with a significant role in translational regulation. CYFIP1/2 (cytoplasmic FMRP interacting protein) proteins are strong candidates for intellectual disability among the FMRP interactors (Hoeffer et al. 2012).

The Fragile X Mental Retardation Gene (FMR1) encoded protein (FMRP) is an RNA-binding protein linked to translational regulation. Dysregulated translation initiation signaling was recently observed in the Fmr1 knockout mouse model of FXS (Handt et al. 2014). As the FMR1 coding region study is not included in normal molecular research, the prevalence of point mutations causing FXS is not well known (Maurin et al. 2014). The deregulation of translation/transport/stability of these mRNAs has a cascading effect on many pathways in the absence of FMRP, resulting in the final phenotype. The proposal of an RNA (fragile X premutation rCGG repeat)-mediated gain-of-function toxicity model for fragile X syndrome has led to several lines of evidence (Li and Jin 2012). Since FMRP is associated with polyribosomes (ribosome clusters, protein-synthesizing molecular machines) and neuron-specific mRNA, it is believed to play an important role in the post-transcriptional control of neuron gene expression (Jayaseelan and Tenenbaum 2012). FXTAS neuropathology consists of moderate brain atrophy and cerebellum degeneration, including middle cerebellar peduncle (MCP) hyperintensity, loss of Purkinje neuronal cells, deep cerebellar white matter spongiosis, Bergman gliosis, and swollen axons (Sellier et al. 2014). Recent studies have provided increased support for the role of FMRP in translational repression via ribosomal stalling and the microRNA pathway. The detection of signalling pathways such as PI3K and mTOR downstream of FMRP-regulating group 1 metabotropic glutamate receptors (mGluR1/5) was explicitly emphasized in neurons. New research also indicates that presynaptic dysfunction and abnormal adult neurogenesis are triggered by FMRP failure (Wang et al. 2012).

Relation between Fragile X syndrome and autism

Different genetic forms of autism are hypothesized to share a similar increase in the cerebral cortex's excitation inhibition (E-I) ratio, triggering hyperexcitability and excess spiking (Langberg 2020). The most common type of heritable mental retardation and the known leading cause of autism is Fragile X syndrome (FXS) (Dölen et al. 2007). FMR1 exposes fresh and

unforeseen clinical presentations and molecular pathways 15 years after its discovery. FMR1 loss of control is a model for neurodevelopmental and behavioral disorders (Jacquemont et al. 2007). including mental retardation, autism, anxiety, and mood disturbance. Recent research by Licznerski et al. indicates that the inner mitochondrial membrane proton leak is elevated by mutant FMRP linked to Fragile-X syndrome, leading to increased metabolism and protein synthesis changes that cause impaired synaptic maturation and autistic behaviors (Mithal and Chandel 2020).

Boys with Fragile X Syndrome (FXS) are at high risk of experiencing signs of attention deficit/hyperactivity and symptoms of the autism spectrum, but their experiences have not been observed over time (Doherty et al. 2020). Despite this convergence, due to inconsistency in diagnosis approaches in the literature, our understanding of autism spectrum disorder symptoms and the seriousness of fragile X syndrome is minimal (Haebig et al. 2020).

A higher degree of depressive symptoms than the other groups of fathers was reported by the fathers of sons or daughters with ASDs. There was a lower degree of pessimism recorded by fathers of sons or daughters with D.S. than by the other fathers. No community variations were found in the paternal coping style. Group variations in paternal depressive symptoms and pessimism were partly linked to differences in paternal age, behavioral issues of the infant, risk of additional disabled children, and maternal depressive symptoms (Hartley et al. 2012). Concerning lower-order (motoric) limited, repetitive behaviours and social approaches, the behavioural phenotype of FXS + Aut and iAut is most similar but varies in more nuanced types of restricted, repetitive behaviours and some behaviours of social response. Such results demonstrate the general phenotypic variability of autism and its unusual presence in an etiologically distinct condition (Wolff et al. 2012). Matrix metalloproteinase 9 (MMP-9) is one of the proteins elevated in FXS, and in the Fmr1 knockout mouse model of FXS, minocycline decreases excess MMP-9 activity. Via randomized therapy trials, both minocycline and mGluR5 antagonists are currently being tested in patients with FXS. In around 10% of males and 2-3% of females, premutation (55-200 CGG repeats) may also lead to the mechanism of autism (Wang et al. 2010). There are many advantages to researching the occurrence and stabilization of autism in infants with FXS, such as clarifying the fundamental causes of autism development in FXS and strengthening similarities and distinctions between co-morbid FXS with autism and I.A. Infant studies in both I.A. and FXS were explored as well as findings and consequences for practice and future research (McCary and Roberts 2013).

Molecular Diagnosis of Fragile X syndrome

Prenatal Diagnosis was based on cytogenetic detection of the fragile X chromosome acquired through cordocentesis in cultured amniotic fluid, chorionic villus cells, or fetal blood. The prevalence of misdiagnosis is around 5% due to unusual false positive and false negative diagnoses more commonly (Tassone 2015). Although with some limitations, southern blot and PCR analysis were replaced by cytogenetic analysis, which was the method of Diagnosis in the early 1990s (Sofocleous et al. 2009).

Molecular and immunocytochemical methods are used in diagnostic approaches. For most laboratories, Southern blot, which makes it possible to detect mutations and assess methylation status in a single test, remains the technique of choice (Gold et al. 2000). The combined use of PCR to amplify normal- and premutation-length alleles and Southern analysis to detect fully expanded alleles and evaluate methylation is required for unequivocal molecular characterization of the FMR-1 triplet expansion region. A simplified molecular diagnostic test based on fluorescent methylation-specific PCR may be an effective alternative or complement to Southern blot analysis for diagnosing Fragile X syndrome (Zhou et al. 2006). In three families, the pfxa3 probe confirmed the cytogenetic Diagnosis, rediagnosing the other three as

non-fragile X. A further two families had the clear expression of a separate fragile site susceptible to folate, FRAXE, similar to FRAXA, but not associated with fragile X syndrome and not detectable by the pfxa3 probe. Subsequent referrals were obtained from additional family members or members of new families for whom related markers did not predetermine carrier status. In these 222 additional cases, a direct diagnosis of pfxa3 for the 135 females was confirmed by dose review with the control probe (Mulley et al. 1992). Changes in the repeat length of FMR1, such as full mutation and premutation, could then be observed. Therefore, the proposed standardization has proven successful in diagnosing FXS, encouraging families to obtain adequate genetic counseling (Gigonzac et al. 2016). It is best practice to use a tool that detects the full range of expansions when examining relatives (including prenatal Diagnosis) in a family with any known fragile X condition due to expansion. The study must state that rare cases of point mutation or deletion can not be identified while testing the FMR1 gene in population screening or rare cases of CGG expansion mosaicism (MoMN), if the tool used, can not detect the full range of expansions (Biancalana et al. 2015).

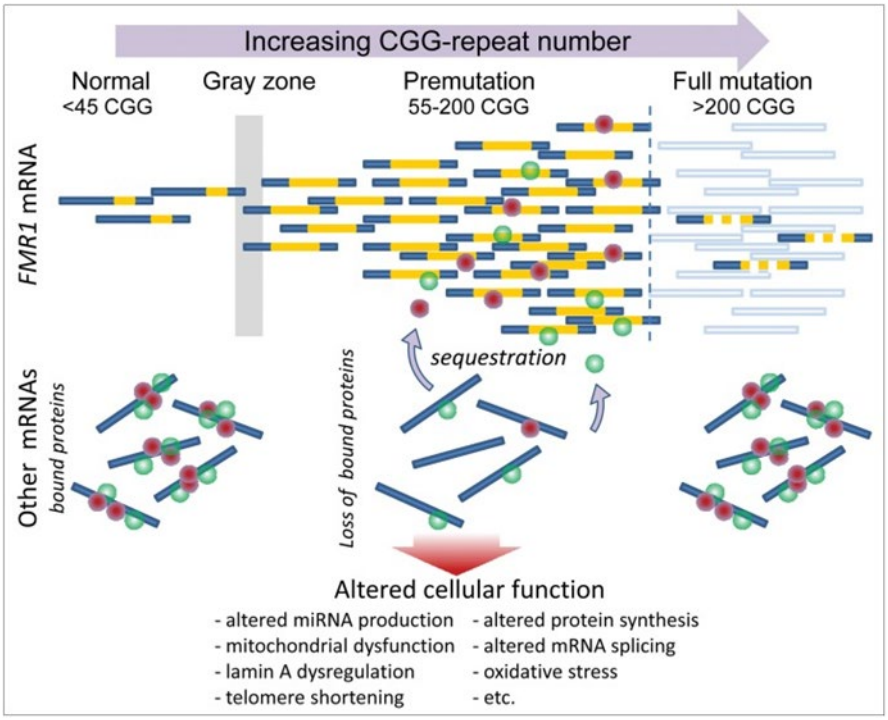

Figure 1. With increasing CGG-repeat length (gold segments) in the premutation range, FMR1 mRNA levels rise and undergo a transition to dramatically decreased levels in the full mutation range due to the FMR1 promoter region hypermethylation.

In some instances, methylation mosaicism in the full mutation spectrum results in the continued development of low-to-moderate mRNA levels. RNA toxicity in the premutation range is thought to occur by direct sequestration of one or more RNA binding proteins that would normally be connected by direct sequestration of one or more RNA binding proteins with other mRNAs binding to the expanded CGG-repeat portion within the FMR1 mRNA. In turn, sequestration contributes to the loss of those proteins' normal function(s), which may include, among other functions, splice modulation, and control of the development of miRNA. It is suspected that RNA processing dysregulation contributes to different types of downstream cellular dysregulation (Hagerman et al. 2012).

Epigenetics of Fragile X syndrome

In complex processes, such as genomic imprinting and transcriptional regimen regulation, epigenetic modifications play a role. Moreover, these modifications are important for optimal brain growth and behavioral performance. Indeed, aberrant epigenetic marks play a part in the etiology of many neurodevelopmental disorders, some of which we still do not completely understand (Doherty and Roth 2020).
In the 5 'region of the fragile X mental retardation1 (FMR1) gene, the primary molecular defect in this disorder is the expansion of a CGG repeat, leading to de novo methylation of the promoter and inactivation of this otherwise normal gene, but little is understood about how these epigenetic changes occur during development (Hecht et al. 2017). Most FXS patients have an expansion over 200 repeats of (CGG)n sequence ("full mutation" (F.M.)) located in the 5′UTR of the FMR1 gene, resulting in local DNA methylation (methylated "full mutation" (MFM)) and epigenetic silencing. The absence of the FMRP protein is responsible for the clinical phenotype of FXS (Tabolacci et al. 2020).
Gene silencing and the degradation by DNA methylation and chromatin remodeling of gene products (Smail 2016, Smail 2019, Shitik et al. 2020, Smail et al. 2022, Smail 2023). The restrictive H3K9 methylation mark is enriched in FXS patient cells by the FMR1 gene and is, therefore, a possible drug target. However, its relation to the silencing process is uncertain (Kumari et al. 2020). To control cell fate decisions, neural development includes orchestrating complex modifications in gene expression. This control is highly affected by epigenetics, not specifically clarified by genomic knowledge alone (Salinas et al. 2020).
Epigenetics research applications are evaluated, including opportunities to map and modify epigenetic marks (Smail et al. 2022). The possibilities for epigenetic and noncoding RNA manipulation-based therapies (Henshall 2020). Silencing the FMR1 promoter through an epigenetic process involving CGG repeat DNA methylation and the regulatory regions surrounding it. The reduction in FMR1 transcription is related to the loss of the FMR1 protein needed for normal brain growth (Kraan et al. 2019). Given the lack of sufficient cellular and animal models that can completely recapitulate the molecular features characteristic of disease pathogenesis in humans, the timing and mechanisms of FMR1 epigenetic gene silencing and repeat instability are far from being understood (Abu and Eiges 2019). More than ~200 CGG repeats result in transcriptional silencing, and the FMR1 encoded protein, FMRP, is absent in the 5' untranslated portion of the FMR1 gene. FMRP is an activity-dependent RNA-binding protein that regulates the transport and translation of various mRNAs in the brain (Kumari et al. 2019). Methylation mosaicism individuals produce more FMRP than individuals with complete mutation alleles completely methylated. Besides, CGG repeat numbers in the premutation range and FMRP expression are inversely related; hence in addition to complete mutation alleles, individuals with size mosaicism who bear premutation alleles will also likely produce more FMRP than non-mosaic individuals. Elevated FXS FMRP levels are associated with fewer clinical symptoms and positively correlate with I.Q. (Rajaratnam et al. 2017).

Gene therapy of Fragile X syndrome

Unquestionably, gene therapy is the definitive way to treat genetic disorders and potentially cure them. In theory, a simple idea in theory, even when directed to easily accessible somatic cell systems, has proven difficult to realize in practice. Gene therapy for diseases in which the target organ is the central nervous system (CNS) poses much greater challenges, and multiple vectors and approaches to brain delivery are under study (Rattazzi et al. 2014). Since no existing clinical therapies are directly aimed at the underlying neuronal defect due to the absence of

FMRP, new effective therapeutic methods may be opened up (d'Hulst and Kooy 2009). The research community agreed to rethink the methods and procedures used, introducing improvements in both the preclinical and the clinical arenas in the face of disparities in expertise and the lack of therapies for FXS patients. This specific problem discusses some of the changes being made to find appropriate therapies for FXS in the field (Kumari and Gazy 2019).
In identifying therapeutic strategies and repurposing medicines for neurological disorders, data show a good value of transcriptome-based computation and suggest trifluoperazine as a possible treatment for FXS (Ding et al., 2020). Despite comprehensive studies using animal models, understanding how FMRP controls human brain development and function remains a major challenge. Human pluripotent stem cells (hPSCs) provide powerful platforms to research human disease processes and test possible therapies. Genome editing, especially the CRISPR/Cas9-based method, is highly effective (Zhao and Bhattacharyya 2020).
In the mouse model of fragile X syndrome, numerous studies have examined the efficacy of adeno-associated viral (AAV) vectors. AAVs have been used to express a fragile X mental retardation protein (FMRP) absent or highly reduced in the condition. These studies have shown several efficiencies in various experiments, from absolute correction to partial rescue to no effect (Hampson et al. 2019). Neurodevelopmental conditions such as Rett Syndrome, Fragile X, and autism still face major hurdles to overcome before the effects of viable human gene therapy can be considered (Gray et al. 2013). offers the first evidence of the theory that gene therapy in the FXS mouse model can correct particular behavioral anomalies (Gholizadeh et al. 2014). Guide RNA-mediated CRISPR-Cas nucleases are a powerful mammalian genome engineering technology. In cell-based models, CRISPR-Cas9-dependent editing of mutated genes causing Huntington's disease and fragile X syndrome has recently been accomplished, heralding the first step towards transforming this technology into viable neurological disease therapeutics (McMahon and Cleveland 2017). Several recent studies suggest minocycline as another possible route of FXS clinical treatment in addition to the promise of mGluR5 pathway therapies (Tabolacci and Chiurazzi 2013). The potential application of selective treatment of genetic disorders through epigenetic methods Clinical studies are underway to transfer outcomes to humans in animal models of FXS, posing complicated problems with the design of trials and outcome measures to test cognitive improvement that may be correlated with therapy (O'Donnell and Warren 2002). Recent trials of novel FXS therapies have highlighted several challenges, including subpopulations with possibly differential therapeutic responses, the lack of specific outcome measures capturing the full range of improvements, A lack of biomarkers that can control whether a particular mechanism is sensitive to a new drug and whether the response is associated with clinical improvement in patients with FXS (Jacquemont et al. 2014). In reversing cellular and behavioral phenotypes and restoring proper brain connectivity in mouse and fly models, the use of metabotropic glutamate receptor (mGluR) blockers and gamma amino-butyric acid (GABA) agonists is successful (Hagerman 2012).
Technologies that can safely edit genes in the brains of adult animals may revolutionize the treatment of neurological diseases and the understanding of brain function. Here, they demonstrate that intracranial injection of CRISPR-Gold, a CRISPR-Cas9 ribonucleoprotein nonviral delivery vehicle, can edit genes in multiple mouse models in the brains of adult mice. CRISPR-Gold can provide ribonucleoproteins for both Cas9 and Cpf1 and can alter all major cell types in the brain, including neurons, astrocytes and microglia, with undetectable levels of toxicity at the doses used (Lee et al. 2018). Multiple pharmacological and genetic manipulations that target receptors, scaffolding proteins, kinases, and translational control proteins can rescue neuronal morphology, synaptic function, and behavioural phenotypes in FXS model mice, presumably by reducing excessive neuronal translation to normal levels (Richter et al. 2015). The results inspired the introduction of clinical trials in patients. The targeted pathways converge in part with those of related neurodevelopmental disorders, raising hopes that the

treatments developed for this specific disorder might be more broadly applicable (Braat and Kooy 2014). Such strategies have led to the development of drugs that are now in clinical trials most promisingly. The research shows how progress in understanding disorders such as FXS has led to a new age in which molecular therapy for neurodevelopmental disorders has become possible (Wijetunge et al. 2013). In addition, S6K1 deletion prevented immature dendritic spine morphology and multiple behavioral phenotypes, including social interaction deficits, impaired novel object recognition, and behavioral inflexibility. The results support the model that the primary causal factor in FXS is dysregulated protein synthesis and that normal translation restoration will stabilize peripheral and neurological function in FXSS (Bhattacharya et al. 2012).

The prevention and care of FXS would result from concerted campaigns, between government medical professionals and the public, in multiple arenas. A specialized clinic and research center is urgently necessary to provide the latest information to Chinese medical practitioners. More publicity and education are needed to provide awareness and resources to individuals with FXS and their families (Niu et al. 2017). Current research indicates that this chromosomal mutation is correlated with a host of other concerns related to education, including learning difficulties, attention deficit disorders, speech and language deficiencies, autism traits, and behavioural disorders. A summary of the distinctive inheritance pattern demonstrates why milder manifestations of the syndrome are now being recognized and educational strategies are being applied (Santos 1992). The proportion of young people with FXS who comply with fundamental recommendations in preventive care guidelines varies depending on health status and demographic factors. For certain classes, this proportion may be increased, particularly in cases of influenza vaccination and physical activity (Gilbertson et al. 2019). SXF molecular prenatal diagnosis is simple and 100 percent accurate, although it is complicated from a scientific point of view and involves using different molecular techniques. The low rate of mutations found guarantees offspring from the therapeutic point of view, while molecular studies do not predict mental status' in either girl with full mutation or children with permutation (Tejada 2001).

Conclusions

From this review, I conducted the following conclusions:

The development of genetic diseases and traits linked to FMR1 gene mutations is known as fragile X syndrome. A scientist is currently focusing on a cutting-edge method for diagnosing and treating Fragile X syndrome. The FMR1 genes are silenced and inactivated by two important epigenetic mechanisms. DNA methylation and remodelling of the chromatin. Future medical care is possible thanks to gene therapy.

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