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Research Article

Modulation of Serum Inflammatory Pattern, Oxidative Stress, Selected Neurotransmitters in Cerebral Cortex of Alloxan Diabetic Rats: Role of Curcuminoids and Fish Oil as Therapeutic Agents

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ABSTRACT
The current study aimed mainly to configurate any changes in selected neurotransmitters pattern, some inflammatory markers , oxidative stress in the brain of alloxan diabetic rats and to illustrate any relation between neurotransmitters activity and the inflammation degree . Effect of Fish oil and curcuminoids either individually or in association form were tested as neuroprotective agents. Materials and Methods: Fourty adult male western albino rats were rendered diabetic by alloxan administration ,then sub classified into five groups ,the first one received no drugs , served as diabetic control (DC) . The second one received Fish oil 50 mg /kgbody weight , the Third one received curcuminoids 50 mg/kg while the fourth one received Fish oil in association with curcuminoids daily for 60 days using the same doses used before. Another group of normal rats (10) was allocated , received no treatment and served as normal group. Results : Compared to normal group we have observed an increased level of MDA , serum IL-6 and CRP along with of GSH , DA , AchE and GABA decrease in the cerebral cortex of alloxan diabetic rats .Fish oil , curcuminoids either alone or in combination form have reversed these Biomarkers to certain degree.

Keywords: dopamine, acetyl cholineeasterase, gamma amino butyric acid, malondialdhyde, reduced glutathione, interleukin-6, C-reactive protein, poly unsaturated fatty acids, central nervous system

INTRODUCTION

Diabetes Mellitus is a syndrome characterized by chronic hyperglycemia and disturbance of carbohydrate, fat and protein metabolism along with absolute or relative deficiency in insulin secretion or insulin action[1] . Persistent hyperglycemia in diabetic patients and despite appropriate therapeutic measures may leads to several complications including retinopathy, nephropathy and neuropathy[2]. Chronic hyperglycemia therefore induce certain pathological characters of both the peripheral and central nervous systems[3]. Several factors are implicated in the pathogenesis of both peripheral neuropathy and CNS disease in diabetics like Oxidative stress, abnormal lipid metabolism, impaired vascular reactivity with reduced blood flow, and neuro inflammation[4].

Different studies have shown that inflammatory markers in blood like C Reactive Protein (CRP), IL-6, are elevated significantly in diabetic population[5], contributing to diabetic complications specifically atherosclerosis. High CRP may be a marker of oxidative stress on the endothelium of diabetic patients, to the extent that higher levels of serum CRP and other inflammatory markers in a normal population represents an indicator for future development of diabetes[6], additionally it may correlate with the severity, complications and degree of diabetic control[7] . High CRP levels are linked also with the presence of metabolic syndrome, association of diabetes with hypertension and other metabolic disturbance[8]. Increased oxidative stress within the cell can lead to activation of the poly (ADPribose) polymerase (PARP) pathway, which regulates the expression of genes involved in promoting inflammatory reactions and neuronal dysfunctions[9]. Glutathione (GSH) represents a peroxide scavenger that compensates the reduced effect of catalase contributing to maintain the normal antioxidant defense status[10]. Certain study indicated before that diet supplemented with fish oil enriched in omega-3 fatty acids especially eicosapentaenoic acid (EPA) and decoscahexanoic acid (DHA) has profound beneficial health effects against various diseases [11] Polyunsaturated omega 3 fatty acids can exert certain effects on the structure, biochemistry and physiological function of the brain where all the brain cells and organelles are enriched with W3 polyunsaturated fatty acids12. The alpha linolenic acid is an essential omega 3 fatty acid can control certain neurosensory and higher functions such as learning. Therefore any quantitative decrease of these fatty acids in the brain results in impairment of membrane function activity of enzymes, receptors and transporters[13]. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) can affect certain

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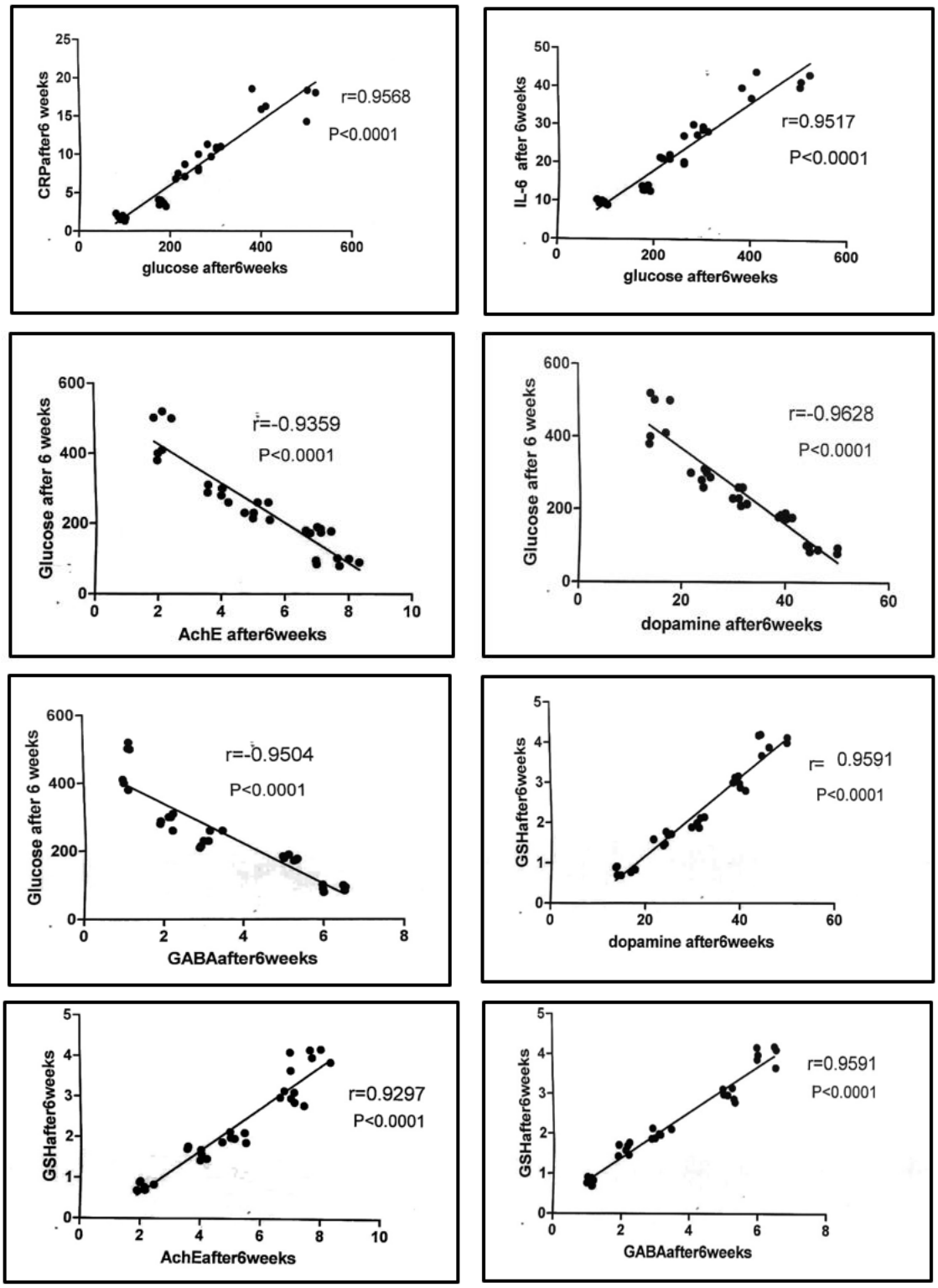

Graph I-VIII: Correlation Studies

gene expression in the brain such as synaptic plasticity, cytoskeleton and membrane association, signal transduction ion channel formation, energy metabolism and regulatory protein[14,15]. Docosa hexaenoic acid (DHA), a component of fish oil is a powerful therapeutic agent which can protect brain tissue , help its repair mechanism and its return to the normal status[16].

Last year certain attempts have been done to enhance the neuroprotective efficacy of FO through its combination with antioxidants/phytochemicals[17] like epigallocatechin-3-gallate (EGCG) and demonstrated certain benefits[18,19]. Curcumin,/curcuminoids a yellow pigment from Curcuma longa, which exhibits a powerful antioxidant, anti-diabetic, anti-inflammatory and anti-cancer properties has

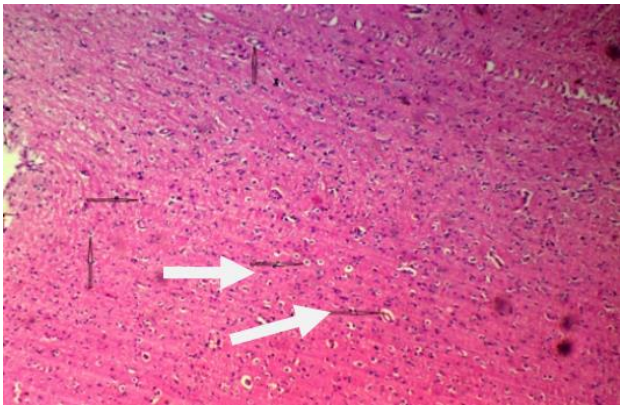

Figure 1: Normal: A Photomicrograph of cerebral cortex of normal brain tissue formed of stellate and pyramidal neuronal all surrounded by eosinophilic neurofibrillary material (Hematoxylin & Eosin X 200).

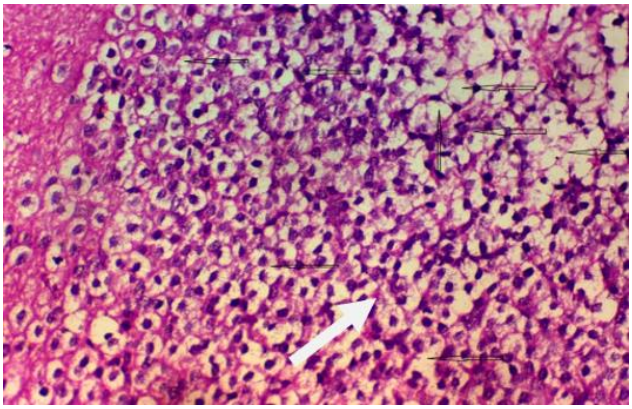

Figure 2: Diabetic edema: A photomicrograph of cerebral cortex of the brain of diabetic rat showing marked area of edema around the neuronal cells(H&E X 400).

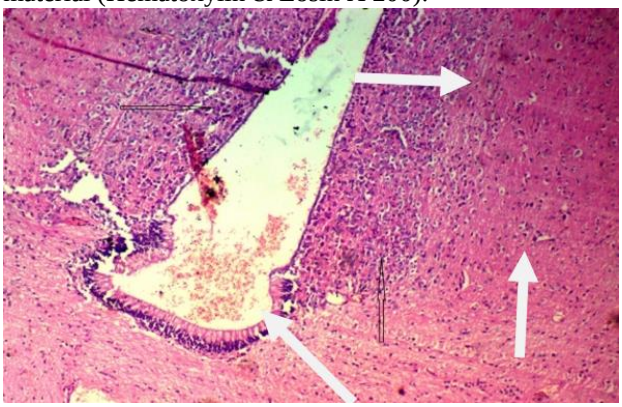

Figure 3: Diabetic+inflammation: A photomicrograph of cerebral cortex of diabetic brain tissue showing aggregates of inflammatory cells around the ventricular wall(H&E X 200).

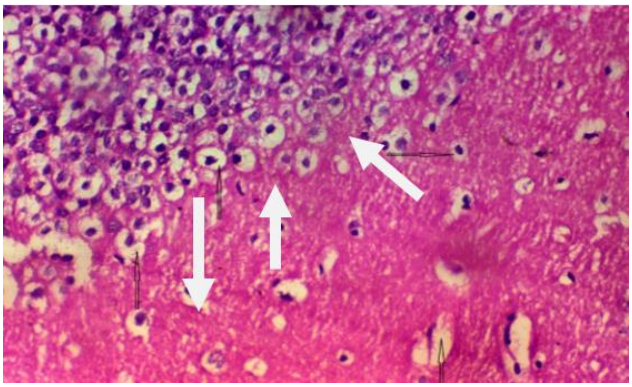

Figure 4: Diabetic edma+fish oil: A Photomicrograph of cerebral cortex of the brain of diabetic rat after treatment with fish oil showing mild improvement of edema around the neuronal cells(H&E X 400).

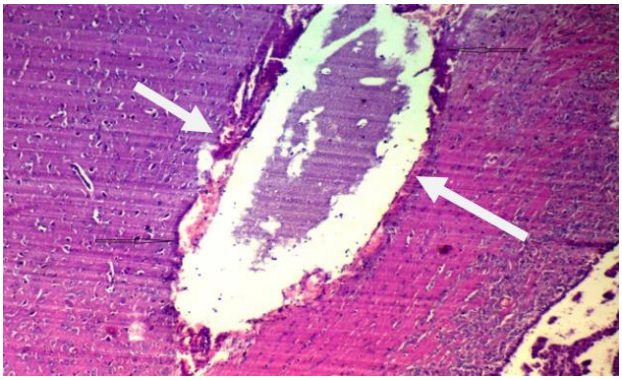

Figure 5: Diabetic inflmmation+fish oil: A photomicrograph of cerebral cortex of diabetic brain tissue after treatment with fish oil showing moderate aggregates of inflammatory cells around the ventricular wall (H&E X 200).

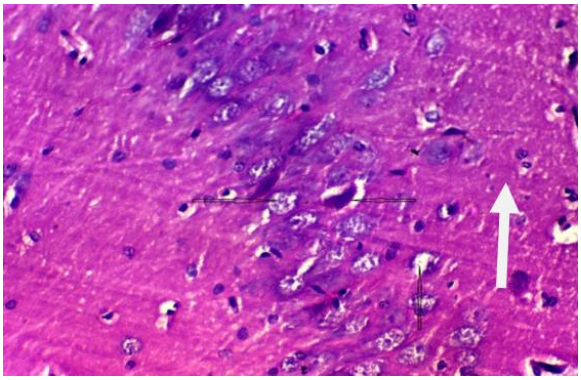

Figure 6: Diabetic edema +curcuminoide : A Photomicrograph of the cerebral cortex of the brain of diabetic rat after treatment with curcuminoide showing that moderate reduction of edema fluid around neuronal cells (H&E X 400).

illustrated neuro protective potential[20,9] . Previous study also indicated that curcumin can antagonize the deficit of glucose energy metabolism or oxidative stress related to cognitive impairment associated with diabetes . It can modulates also the expression of various molecular targets such as transcription factors, enzymes, cytokines, cell cycle proteins, receptors and adhesion molecules[21].

Present work therefore aimed mainly to configurate any modulatory effect of fish oil either alone or in combination with curcuminoids on certain brain neurotransmitters profile, oxidative stress and other inflammatory markers. This may illustrate the potential of these natural agents and may presented it as new candidate for therapeutic agents .

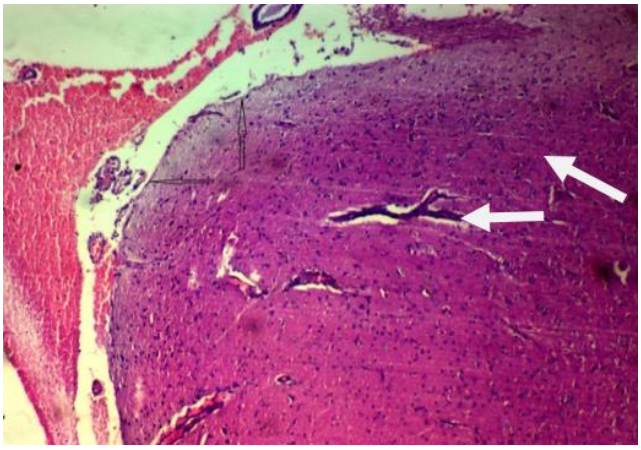

Figure 7: Diabetic inflammation +curcuminoide: A photomicrograph of cerebral cortex of the brain of diabetic rat after treatment with curcuminoide showing very few of inflammatory cells around the ventricular wall (H&E X 200).

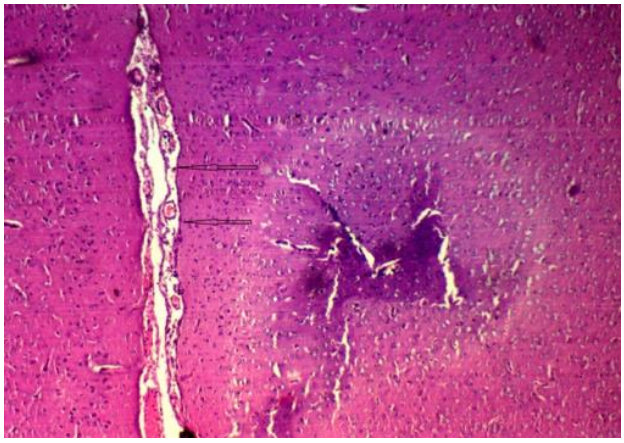

Figure 8: Diabetic inflammation +combination treatment : A photomicrograph of cerebral cortex of diabetic brain tissue after treatment with combination of curcuminoide and fish oil showing disappearance of inflammatory cells around ventricular wall (H&E X 200).

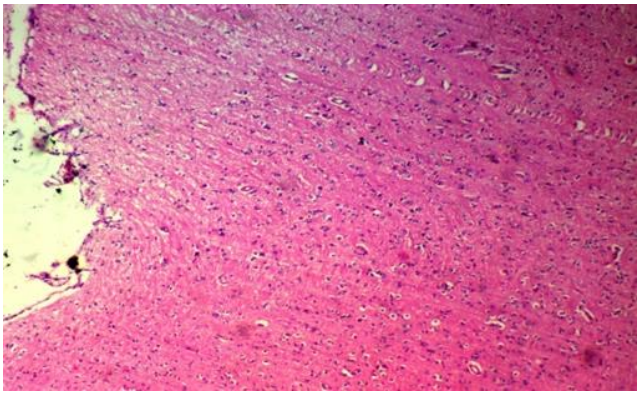

Figure 9: Diabetic edema+combination: A photomicrograph of cerebral cortex of brain of diabetic rat after treatment with combination of curcuminoide and fish oil showing return of the cerebral cortex structures to the normal appreance.

MATERIALS AND METHODS

Materials

Fish oil and was purchased from Arab Co. for gelatin and pharmaceutical products , cairo-Egypt

Preparation of –curcumenoids

Curcumenoids were extracted from Curcuma Longa (Family Zingiberaci) according to Piper *etal* [22] .The extract was loaded on silica gel column (60-120 meshes), eluted with mixture of methanol: chloroform by the ratio of 9: 1 respectively.The active fraction curcumenoids (yellow colour) is collected, evaporated under reduced pressure by rotary evaporator to render it alcoholic free and stored at 2-8ºC [23] .

Methods

All experiments were approved by the ethical committee of the faculty of pharmacy,zagazig university. Experimental design and animal handling were performed according to the guidelines of the ethical committee of the faculty of pharmacy , zagazig university for animal use and in accordance with the recommendations of the weatherall report.

Animals

Male albino rats of 180-230 g body weight were used in the present study. The animals were firstly allowed to acclimatize for 2 weeks . They were housed individually in separate cages under 12 hours light and 12 hours dark periods. Rats were fed rodent chow (ELnasr pharmaceutical company, cairo) and allowed free excess of drinking water . The protocols for animal experimentation and the handling of animals were in accordance with the animal welfare act and the guide for the care and use of laboratory animals established by Zagazig university, Zagazig-Egypt .

Induction of Diabetes Mellitus

A single dose (120mg/kg) of freshly prepared solution of Alloxan Monohydrate (dissolved in Normal Saline, Citrate buffer, pH 4.5) was administered I.P to overnight fasting rats for induction of diabetes mellitus[24]. Control rats were similarly injected with normal saline. Fasting blood glucose level was checked after 48-72 hours. Rats which achieve a blood sugar level > 200mg/dl were selected as diabetics, processed later according to the following classification.

Exprimental design

Group1: rats received chow diet only , expressed as normal group.

Table 1: The changes in blood glucose level and body weight in different groups after treatment with fish oil and curcuminoide for 3-weeks:

Time	Parameters	control	Diabetic	Fish oil	curcuminoide	Fish oil + curcuminoide
3 weeks	Body weight (gram)	228.8±5.7	135.3±7.1#	184.8±6.7*	199.3±6.7*a	218.7±7.7*ab
	Serum glucose (mg/dl)	92±8.6	452.0±61.7#	289.7±17.91*	234.2±21.54*a	180.2±6.39*ab
	IL-6 pg/ml	9.55±0.5	31.43±2.0#	19.35±0.5*	15.35±0.8*a	11.48±0.38*ab
	CRP ng/ml	1.78±0.3601	9.56±.8#	7.11±0.3*	5.98±0.2*a	3.66±0.2*ab
6 weeks	Body weight (gram)	208.5±9.0	153.0±7.0#	167.7±8.6*	182.2±8.5*a	198.5±5.3*ab
	Serum glucose (mg/dl)	91.50±7.9	401.8±58.2#	309.7±39.2*	260.2±11.7*	193.7±9.2*ab
	IL-6 pg/ml	9.55±0.5	41.03±2.5#	28.52±1.1*	20.95±0.8*a	13.25±0.6*ab
	CRP ng/ml	1.78±0.3	16.93±1.7#	10.60±0.6*	7.70±0.71*a	3.70±0.3*ab

Table 2: The changes in inflammatory markers after treatment with fish oil and curcuminoidefor 6-weeks:

S. No	Parameters	Control	Diabetic	Fish oil	Curcuminoide	Fish oil + curcuminoide
1	MDA nmol/g tissue	11.25±.6	50.17±2.9#	34.12±2.6*	26.1±1.3*a	14.98±1.0*ab
2	GSH µmol/g tissue	3.99±.2	.79±0.9#	1.60±0.1*	1.99±0.1*a	2.98±0.1*ab
3	GABA ng/g tissue	6.24±.2	1.08±.0#	2.08±.1*	3.07±.2*a	5.16±.1*ab
4	AchE ng/g tissue	7.60±.5	2.09± .11#	3.08±.2*	5.12±.3*a	7.01± .2*ab
5	DA ng/g tissue	46.62±2.7	15.18±1.7#	23.95±1.2*	31.12±.9*a	39.75±.9*ab

Significantly different from normal group.
* significantly different from diabetic group.
a significantly different from fish oil group.
b significantly different from curcuminoide group.

Group2 : rats received alloxan only ,and served as diabetic control.
Group3: Alloxan diabetic rats , received fish oil orally (50mg/kg body weight) daily.
Group4: Alloxan diabetic rats , received curcuminoids orally (50mg/kg body weight) daily.
Group5: Alloxan diabetic rats treated , received fish oil and curcuminoids using the same doses before daily for 6 weeks

During the experimental period (6 weeks), body weight, blood glucose, food and water consumption and physical examinations were determined at regular intervals. The dosage was adjusted every week according to any change in body weight to maintain the dosage state. At the end of the treatment periods (3 and 6 weeks), the rats were fasted overnight (10h), blood samples were collected and sera were prepared. Rats after 6 weeks treatment were n sacrificed under diethyl ether anesthesia, the brain was dissected out quickly, cerebral cortex was taken , rinsed with ice-cold saline and homogenized instantly on ice using buffer . Tissue homogenate and sera were kept at −80◦C for further analysis.

Biochemical analysis

Blood glucose was determined using commercial kits provided by Spinreact Kits ,Barcelona ,Spain . Malondialdehyde as a marker of lipid peroxidation and Glutathione (GSH) were determined using Bio-Diagnostic kits ,Cairo ,Egypt. C-reactive protein (CRP) was determined using BD Biosciences ELISA Kits, USA. Serum IL-6 was determined using Rat IL-6 Elisa Kits and supplied by biological laboratories inc (IBL-USA). AchE was determined using ELISA kit, clould – clone corp, USA. GABA was determined using rat GABA elisa kit, Eiaabco Co., USA. DA was determined using ELISA technique, live Science, Inc, USA.

Statistics

All values were expressed as mean ± SD of six animals per group. Data were analyzed using one way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple pair wise comparisons between the various treated groups. Values with $P<0.05$ were considered as statistically significant.

RESULTS

Blood sugar and inflammatory markers

Alloxan administration induced significant increase in blood glucose level along with body weight decrease ,Serum IL-6,CRP levels showed also significant increase after 3 and 6 weeks .Diabetic rats , received fish oil , curcuminoids either alone or in combination form demonstrated significant decrease of blood glucose ,IL-6 and CRP (table 1).

Cerebral cortex tissue

Alloxan administration induced significant decrease in GSH, dopamine AchE, GABA while MDA content showed significant increase, Treatment with fish oil or curcuminoids and their combination for 6 weeks significantly decreased MDA content while GSH, Dopamine, AchE and GABA levels demonstrated significant increase as compared to diabetic control (table 2).

Correlations study

Serum glucose level showed positive correlation with CRP, IL-6 after 6 weeks of Diabetic induction ($r=0.95$, $P\leq 0.001$). However Negative correlation was observed between serum glucose level and AchE, Dopamine and GABA ($r= - 0.93$, -0.96 and -0.95, $P \leq 0.0001$) respectively. GSH showed in addition positive correlation after 6 weeks with Dopamine, AchE and GABA ($r= 0.95$, 0.92 and 0.95, $P\leq 0.0001$) respectively

Histopathological Study

Cerebral cortex of the diabetic rats demonstrated marked area of edema around the neuronal cells and aggregation of inflammatory cells around the ventricular walls (Figures 2 ,3) as compared to normal rats (Figure 1).

Fish oil group showed mild improvement of edema and moderate aggregation of inflammatory cells around the ventricular walls (Figures 4, 5) while Curcuminoids group showed moderate reduction of edema and very few inflammatory cells (Figures 6 ,7). Rats which received combination of Fish oil and curcuminoids showed disappearance of inflammatory cells around the ventricular walls and illustrating to certain extent normal appearance (Figures 8, 9)

DISCUSSION

As reported before chronic hyperglycemia is the major initiator for diabetic vascular complications. Enhanced poly hexosamine pathways, activation of protein kinase c (pkc), oxidative stress and over-production of advanced glycation end products (AGEs) may collectively contribute for such complications[25].

The interaction of AGEs with their RAGE receptors located on many cell types may alter intra cellular signaling, gene expression, release of pro-inflammatory molecules (cytokines), free radicals and all are mostly responsible for the subsequent diabetic complications.

Among the most well-known pro-inflammatory cytokines are interleukins (IL-1, IL,6, IL-18) and tumor necrosis factor-alpha (TNF-α)[26].

Neuronal disorders can also induced in subsequent to oxidative stress[27] where neurophysiological, structure changes in the brain and cocgnition defects are additionally produced[28,29]. It seems also probable that neuronal injury may be attributed to excessive generation of free radicals coming from the auto oxidation of elevated intracellular glucose level .Increased lipid peroxidation along with inhibition in enzymatic and non-enzymatic antioxidants level, leading to B-cell damage are additional factors[30].

Present work in confirm demonstrated an increase in brain MDA content in diabetic rats and in agreement with previous study[31]. Increased lipid peroxidation is associated also with GSH decrease in cerebral cortex of diabetic rats and in approval with recent study[30].

Elseweidy et al [23] demonstrated also similar increase of kidney MDA in diabetic renal injury state of experimental rats.

Serum CRP additionally IL-6 as inflammatory markers showed also significant increase as compared to normal group and in agreement with previous findings[32]. Correlation study between hyperglycemia and these inflammatory markers was positive and highly significant. Results of the histological findings may be in confirm where cerebral cortex tissue exhibited marked area of edema around the neuronal cells in addition to the aggregation of inflammatory cells around the ventricular walls of the diabetic control.

Neurotransmitters like dopamine, acetyl chloline, GABA and glutathione are mostly involved in the maintenance of glucose homeostasis[33,34] and are modulated in addition by diabetic state. This is true where the activation and inhibition of different neurotransmitters in diabetic rats are known to be disrupted during the disease

Present study in confirm showed decreased levels of GABA, DA and AchE in cerebral cortex of diabetic rats. Negative correlation was also observed between these neurotransmitters and serum glucose level. AchE, an enzyme found in the synaptic clefts of the cholinergic suspense, cleaves the neurotransmitter acetyl chloline into its constituent's acetate and choline thus limiting the size and duration of the post synaptic potential. It play therefore significant role in ending cholinergic neurotransmission. Its decreased level her in the cerebral cortex of diabetic rats is in agreement with that reported before[35,36].

GABA is the principal inhibitory neurotransmitter in the cerebral cortex which maintains the inhibitory tones that counter balance neuronal excitation, therefore when this balance perturbed, seizures may ensure[37]. Previous report referred to decreased GABA content in the brain of diabetic rats[35] and our results showed also similar findings.

DA is the predominant catecholamine neurotransmitter in the mammalian brain, where it plays multiple roles in the periphery as a modulator of cardio vascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastro intestinal motility[38].

Accordingly the observed decrease of these neurotransmitters may be attributed to monoamines

oxidation through oxygen free radicals in subsequent to induced hyperglycemia. Dopamine biosynthesis may be also affected due to its exposure to mild oxidizing conditions leading to its partial oxidation. This may collectively indicates that any disturbances in neurotransmitters levels like dopamine, serotonin and their oxidation metabolites may be associated with neurodegenerative disease[39].

Our study demonstrated also decreased DA in the cerebral cortex of diabetic rats and in agreement with others[36] which is mostly attributed to higher glucose level, affecting in turn the dopaminergic activities.

Therefore, administration of fish oil, curcuminoide either individually or in combination form resulted in significant decrease of serum glucose, CRP and Il-6. Cerebral cortex contents of MDA showed also significant decrease while GABA, AchE and DA illustrated significant increase as compared to diabetic control.

Curcuminoids effect in general was remarkable than fish oil while their combination effect seems to be superior than each one individually and mostly attributed to certain kind of synergism. Curcuminoids as a natural product are characterized by a variety of pharmacological effects as inhibitor for inducible nitric oxide synthase (iNos) additionally its potential as radical scavenger[40]. In confirm the correlation coefficient between GSH and these neurotransmitters was positive and highly significant

The latter effect is mediated through multiple mechanisms involving inhibition of the activation of various transcription factors such as nuclear factor kappa B (NFKB) , activated protein -1 (AP-1) and peroxisome proliferator activated receptor –γ(PPAR- γ)[41]. Additional mechanism includes down regulation of the production of pro inflammatory cytokines like interleukin -1β(IL-1β)[42].

Regarding fish oil many studies have demonstrated the ability of dietary unsaturated fatty acids to reduce endothelial chemokine expression[43]. One possible explanation relates to the intracellular mediators of NF-KB activation, namely ROS derived from the activation of NADH or NADPH oxidase following cytokine activation. A scavenging effect of O_2 and the presence of NO radical are likely to account for the inhibition of NF–KB activation possibly through enhanced transcription or stabilization of the inhibitor 1-KB[44].

It is conceivable that similar oxygen –scavenging reaction occur with unsaturated fatty acids , preventing in turn O_2 from generating H_2O_2 indeed reduction of cell activation.

Both EPA and DHA (Fish oil contents) have been shown to be ligands for PPAR γ and α exerting an anti-inflammatory effect both *in vitro* and *in vivo*[45].

This effect may be mediated through synthesis inhibition of pro inflammatory molecules such as Il-6, prostaglandins , monocyte cytokine expression and activation of NF-KB via the PPAR dependent pathway[46].

As mentioned before Curcuminoid results may be remarkable to certain extent as compared to fish oil where very few inflammatory cells was observed around the ventricular walls along with moderate reduction of edema. However their combination was superior than individual ones and apparently due to certain kind of synergism between the two. This may be true where cerebral cortex tissues achieved approximately normal pattern along with the disappearance of inflammatory cells as evident from the histogram pattern.

CONCLUSION

Our study has suggested that the combination of fish oil and curcuminoids for treatment of diabetic rats can play a vital role in achieving nearly normal level of the tested neurotransmitters .Its additional potential to alleviate oxidative stress and inflammation are also evident. Thus, curcuminoids and Fish oil have a significant role as a therapeutic agents for the attenuation of diabetic complications in the cerebral cortex. The histological results has offered great support to the biochemical data. Further *In vitro* and clinical studies are highly requested to provide additional outcomes.

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CONFLICT OF INTEREST

Authors have no potential conflicts of interests relevant to this article to be reported . Present work didn't receive any grant or financial support elsewhere.

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