

Role of Oxidative Stress in the Pathogenesis of OCD

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Abstract

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Oxidative stress is one of the mechanisms involved in neuronal damage induced by free radicals which causes membrane lipid degradation which occurs due to interaction of polyunsaturated fatty acids (PUFA). These free radicals cause catalytic destruction of membranes and likely damage vital functions of cell. Oxygen radicals are continuously produced intracellularly by oxidation- reduction reactions. MDA (malondialdehyde) is a product of lipid peroxidation. Oxidative damage of neuronal membrane causes increased MDA levels and impaired activity of SERT.

Keywords: Obsessive compulsive disorder; Serotonin; Malondialdehyde; Reduced glutathione; Free radicals

Introduction

Obsessions are defined as recurrent, persistent, thoughts, images or impulses that are experienced as intrusive & inappropriate. Compulsions are repetitive behaviours or mental acts that the person feels driven to perform in response to an obsession or according to rigid rules [1].

Until the 1850s, obsessive-compulsive phenomena were considered to be a variant of the old notion of insanity. After 1860, organic causal hypotheses for OCD included dysfunctions of the autonomic nervous system and cortical blood supply. In 1880 OCD was included in the spectrum of neuroses. Psychological hypotheses suggested the OCD might result from volitional, intellectual, or emotional impairment, the last of which predominated after 1890 [2]. First description of OCD in childhood was given by Pierre Janet in 1903 who reported on a five year old boy with characteristic symptoms. OCD usually begins in adolescence or early adulthood although it can begin in childhood. Nearly 65% of the patients have the onset before 25 years of age and <15% have onset after 35 years of age [3] (Table 1).

Serotonin (5- Hydroxy Tryptamine)

It's a monoamine neurotransmitter. Biochemically derived from tryptophan, serotonin is primarily found in the gastrointestinal (GI)

| Obsessions | % |
|--|----|
| Fear of contamination | 61 |
| Aggressive thoughts, images and impulses | 43 |
| Need for symmetry | 35 |
| Sexual | 31 |
| Religion | 30 |
| Pathological doubt | 21 |
| Miscellaneous | 40 |
| Compulsions | % |
| Cleaning and washing | 50 |
| Ordering | 41 |
| Repeating | 38 |
| Checking | 18 |
| Hoarding | 7 |
| Miscellaneous | 41 |

Table 1: The following are the common obsessive – compulsive symptoms.

tract, platelets, and in the central nervous system (CNS). Functions of serotonin include the regulation of mood, appetite, sleep, cognitive functions, including memory and learning [4,5] (Figure 1).

Serotonin is the major neurotransmitter in raphe nuclei, which are grouped into nine pairs and distributed along the brainstem, centered around the reticular formation. Axons of neurons in the "caudal" raphe nuclei terminate at deep cerebellar nuclei, cerebellar cortex, spinal cord (Figure 2).

Serotonin is released at terminal synaptic boutons acting at a wide gap of >20 µm synapse and acts to activate 5-HT receptors located on

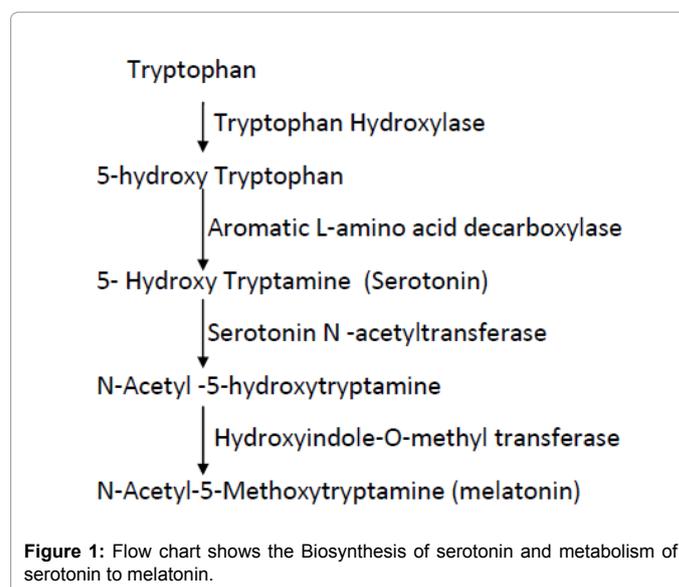


Figure 1: Flow chart shows the Biosynthesis of serotonin and metabolism of serotonin to melatonin.

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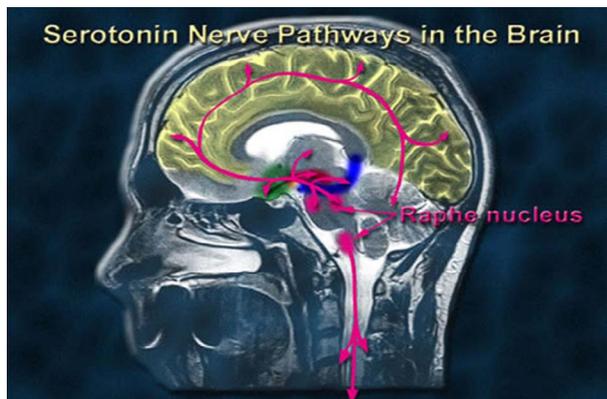


Figure 2: Serotonin Nerve Pathways in the Brain.

the dendrites, cell bodies and presynaptic terminals of adjacent neurons. Serotonin receptors are G protein coupled seven transmembrane (or "heptahelical") receptors that activate an intracellular second messenger cascade except 5-HT₃, which is ligand gated ion channel [6].

SERT spans the plasma membrane 12 times. It belongs to NE, DA, SERT monoamine transporter family. Transporters are important sites for agents that treat psychiatric disorders. Both drugs that reduce the binding of serotonin to transporters (selective serotonin reuptake inhibitors, or SSRIs) and, less often, that increase it (selective serotonin reuptake enhancers, or SSREs) are used to treat mental disorders. About half of patients with OCD are treated with SSRIs. Fluoxetine is an example of a selective serotonin reuptake inhibitor, and tianeptine is an example of a selective serotonin reuptake enhancer. The highly evolutionarily conserved serotonin transporter (SERT) regulates the entire serotonergic system and its receptors via modulation of extracellular fluid serotonin concentrations [7].

The gene that encodes the serotonin transporter is called solute carrier family 6, member 4 (SLC6A4). In humans the gene is found on chromosome 17 on location 17q11.1-q12.

Free Radical Biochemistry

Free radicals are highly reactive molecular species with an unpaired electron; that persist for only a short time of the order of 10⁻⁹ to 10⁻¹² seconds before they collide with another molecule and either abstract or donate an electron in order to achieve stability. In doing so, they generate a new radical from the molecule with which they collided.

An atom is composed of central nucleus with pairs of electrons orbiting around it. Each electron in a pair has an opposite intrinsic angular momentum. Some atoms and molecules have unpaired electrons and these are called free radicals. Free radicals are usually unstable and highly reactive because the unpaired electrons tend to form pairs with other electrons. Free radicals are result of normal cellular metabolism.

Generation of Oxygen Free Radicals

Electron leakage

Major source of free radicals is due to 'electron leakage' from electron transport chain. Normally efficiency on electron transfer reactions is so high that most of oxygen is completely reduced to form water and <2% forms oxygen free radicals.

Normal oxidation reduction reactions

Generation of free radicals is common during normal metabolism. Examples:

- Autooxidation of certain compounds
Adrenaline, thiols, ascorbic acid
- Flavin coenzymes present in peroxisomes (Figure 3).
- Enzymes like xanthine oxidase, aldehyde oxidase and dihydro-orotate dehydrogenase can also generate oxygen free radicals [8].

Exogenous agents

Toxic compounds like carbontetrachloride, exposure to ionizing radiation damages tissues by producing free radicals. Cigarette smoking, alcohol, pollution produce immense high concentration of free radicals. Drugs propafenone, amiodarone are known to produce ROS.

Respiratory burst

Deliberate production of free radicals by the phagocytes with the purpose of destroying pathogens. The most damaging free radicals in biological systems are oxygen radicals especially superoxide (O₂⁻), hydroxyl (OH[·]) and perhydroxyl (O₂H[·]), hydroperoxy (HOO[·]), peroxy (ROO[·]). H₂O₂ (hydrogen peroxide), hypochlorous acid (HOCl), O₃ (ozone), O₂ (singulate oxygen) are non radicals (Figure 4).

Lipid Peroxidation

Refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes,

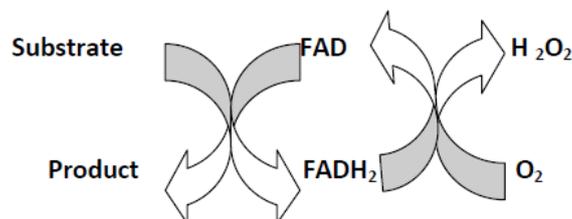


Figure 3: Flavin coenzymes present in peroxisomes.

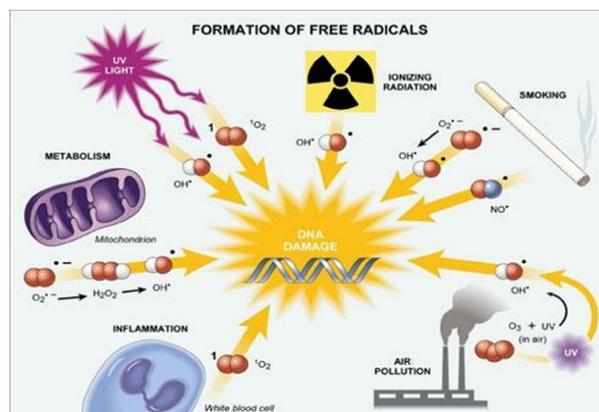


Figure 4: Formation of Free Radicals.

resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene bridges (-CH₂-) that possess especially reactive hydrogens. As with any radical reaction, the reaction consists of three major steps: initiation, propagation, and termination.

Initiation

Initiation is the step in which a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as OH· and [HO₂], which combines with a hydrogen atom to make water and a fatty acid radical.

Propagation

The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxy-fatty acid radical. This radical is also an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and lipid peroxide, or cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

Termination

When a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for there to be a high probability of collision of two radicals. Living organisms have different molecules that speed up termination by catching free radicals and, therefore, protecting the cell membrane. One important such antioxidant is vitamin E. Other anti-oxidants made within the body include the enzymes superoxide dismutase, catalase, and peroxidase (Figure 5).

Malondialdehyde (MDA)

Malondialdehyde or malonaldehyde can be formed during lipid peroxidation of polyunsaturated fatty acids (PUFA), as explained previously under the heading of 'Lipid peroxidation'. MDA is ubiquitous compound produced in substantial quantity in mammalian tissues both as an end product of unsaturated lipid peroxidation and as a side product of prostaglandin and thromboxane biosynthesis. It is also produced by γ- irradiation of carbohydrates [9,10].

Under physiological conditions pH 7.4

MDA exists as an enolate anion (O-CH=CH-CHO), a form that

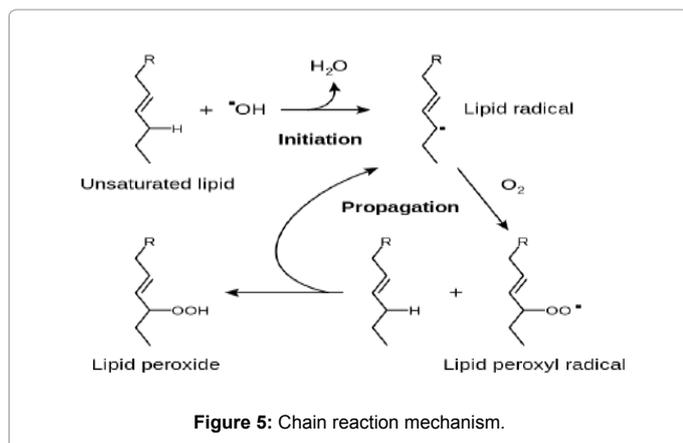


Figure 5: Chain reaction mechanism.

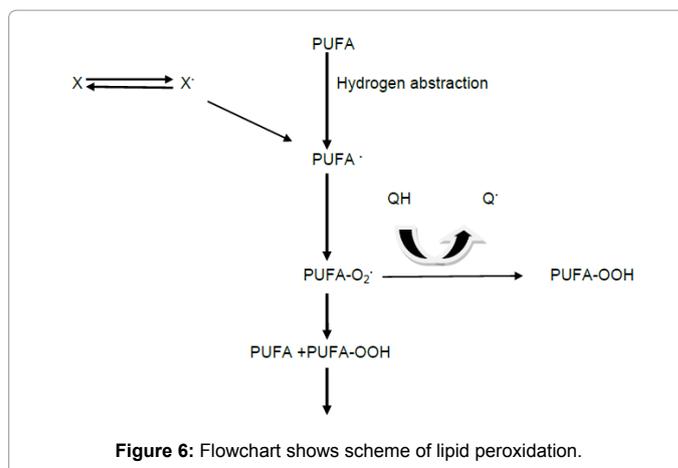


Figure 6: Flowchart shows scheme of lipid peroxidation.

is only fairly reactive, forming Schiff bases with molecules containing a free amine group.

Under more acidic conditions (pH<4), however, β-hydroxyacrolein (HO-CH=CH-CHO) (βHA) is the pre-dominant form. Unfortunately, βHA is a very reactive electrophile capable of reacting in a Michael addition with a number of biologically important nucleophiles (e.g., thiols). Proteins are much more reactive with MDA than free amino acids forming a variety of adducts and cross-links. MDA can also react with DNA bases producing a variety of mutagenic compounds. Further- more, MDA has the potential to induce amino-imino-propen cross-links between complimentary strands of DNA and can also cause the formation of DNA-protein cross links.

MDA has been reported to be toxic, carcinogenic and mutagenic. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine [11-14] (Figure 6).

Reduced Glutathione

It's a water soluble linear tripeptide composed of glutamine, cysteine, and glycine. Referred to as body's master antioxidant. Technically N-L-gamma-glutamyl-cysteinyl glycine or L-glutathione, the molecule has a sulfhydryl (SH) group on the cysteinyl portion, which accounts for its strong electron-donating character. Cysteine is the rate-limiting factor in cellular glutathione synthesis, since this amino acid is relatively rare in food stuffs. It is virtually present in every cell of the body. The highest concentration of glutathione is in the liver, making it critical in the body's detoxification process [15] (Figure 7).

Synthesis of Glutathione

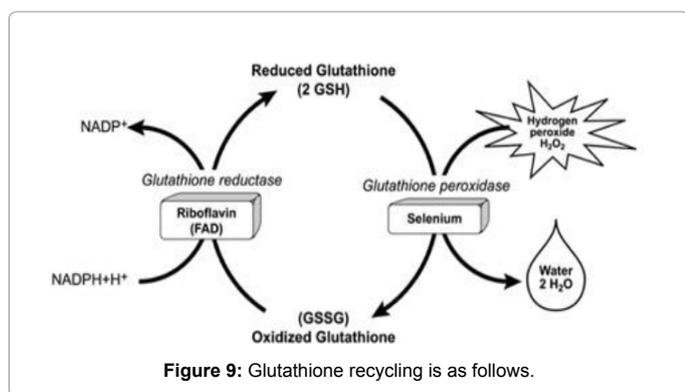
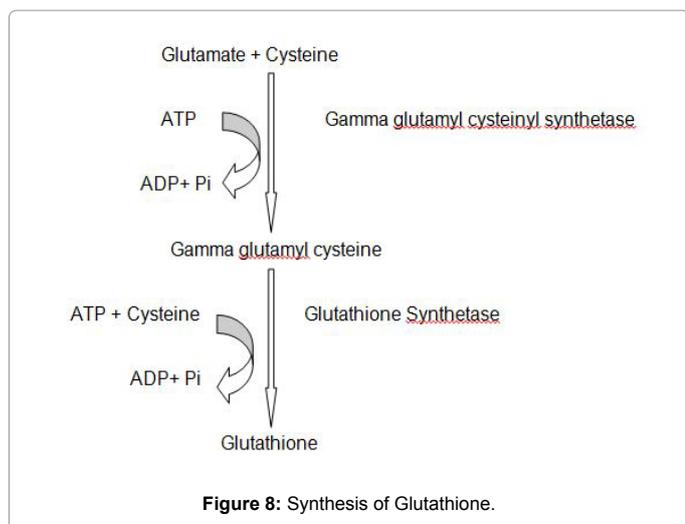
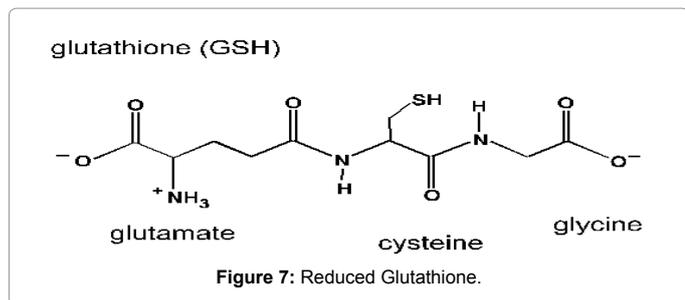
Cells make Glutathione in two adenosine triphosphate (ATP) dependent steps:

First, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme gamma-glutamylcysteine synthetase (glutamate cysteine ligase, GCL). This reaction is the rate-limiting step in glutathione synthesis.

Second, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase (Figures 8 and 9).

Glutathione - Its Multiple Functions

- It is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive



oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms [16].

- Regulation of the nitric oxide cycle, which is critical for life but can be problematic if unregulated [17].

- It is used in metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system, especially the immune system, the nervous system, the gastrointestinal system and the lungs.

- It has a vital function in iron metabolism. Yeast cells depleted of or containing toxic levels of GSH show an intense iron starvation-like response and impairment of the activity of extra-mitochondrial ISC enzymes, followed by death [18].

- Glutathione (GSH) participates in leukotriene synthesis and is a cofactor for the enzyme glutathione peroxidase. It is also important as a hydrophilic molecule that is added to lipophilic toxins and waste in the liver during biotransformation before they can become part of the bile. Glutathione is also needed for the detoxification of methylglyoxal, a toxin produced as a by-product of metabolism.

Materials and Methods

This is a case control study. Ethical affirmations are as follows

Volunteered Research participants: 30 cases of obsessive compulsive disorder and 30 controls (age & sex matched)

- The study has ensured quality and integrity of research.
- I have taken proper informed consent in the language understandable by the patients after explaining the purpose of study and the concept behind the study and risk involved.
- I have maintained the respect, confidentiality and anonymity of my research participants.
- It has been ensured that participants will involve in my study voluntarily and no decisions had been forced upon them.
- I has avoided harm to the participants to the best possible extent; and
- I claim that my research is independent and impartial.

After obtaining informed consent, 30 cases of clinically diagnosed with OBSESSIVE COMPULSIVE DISORDER attending psychiatry OPD were enrolled in the study. Age and sex matched thirty normal individuals were studied as controls. Under all aseptic precautions, 5 ml of venous blood was drawn through phlebotomy using disposable syringe. This sample was distributed in two different vacuitainers: 4 ml in plain vacuitainer and allowed to clot and 1 ml in EDTA coated vacuitainer. After one hour, serum was separated by centrifuging at 2500 rpm for 5 min at room temperature. Separated serum should be free from hemolysis and turbidity.

Estimation of Serum MDA Level

Thiobarbituric acid method for MDA estimation

Serum sample with lipid peroxide is first treated with trichloroacetic acid (TCA) for protein precipitation and then treated with 0.67% thiobarbituric acid (TBA). The mixture is treated for 10 minutes in boiling water bath. One molecule of MDA reacts with two molecules of TBA. The resulting chromogenic adduct is centrifuged and the intensity of color developed in supernatant is measured colorimetrically at 530 nm. MDA levels are expressed in nanomol/ml; (nmol/mL); normal range 2-5 nmol/mL.

Reagents

A. 40% TCA (trichloroacetic acid)

40 gram of TCA in 100 ml of distilled water.

B. 0.67% TBA (Thiobarbituric acid)

Dissolve 670 mg of TBA in 100 ml of distilled water in boiling water bath.

Prepare this reagent freshly.

C. Stock standard MDA (malondialdehyde)

Stock MDA is prepared from 1,1,3,3, tetraethoxypropane

by acid hydrolysis. A solution containing 0.5 mmol of 1,1,3,3, tetraethoxypropane in 50 ml distilled water and 0.5 ml 0.1 M HCL. Is warmed at 50 degree centigrade for 1 hour and the volume adjusted to 100 ml with distilled water. The concentration of free MDA was determined spectrophotometrically at 267 nm using a molar absorption coefficient of 31800 (Table 2).

Mix well and keep the tubes in boiling water bath for ten minutes. cool at room temperature. Centrifuge at 1500 rpm for 5 minutes. Pipette the supernatant and read at 530 nm. The unknown test concentration were plotted for standardization graph.

Method for the Determination of Reduced Glutathione Concentration

Principle

Virtually all the non-protein sulphhydryl groups of RBC's are in the form of reduced GSH. 5',5' DTNB is a disulphide chromogen that is readily reduced by sulphhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration. Estimation of reduced glutathione (GSH) done using DTNB reagent and analyzed within 24 hours of sample collection. Blood levels of GSH expressed in mg/dL. Normal range 17-39 mg/dL.

Specimen collection

Whole blood (3 ml) anticoagulated with EDTA.

Included are normal control sample specimen with each assay.

Reagents

Precipitating solution

1.67 gm glacial metaphosphoric acid + 0.20 gm disodium or dipotassium EDTA + 30 gm NaCl per 100 ml of distilled water

(solution is stable for approximately 3 wk at 4 degree centigrade, stored in deep freezer)

Phosphate solution

0.3 M Na₂HPO₄ prepared in distilled water (42.59 gm of Na₂HPO₄ in 1000 ml DW) stable indefinitely at 4 degree centigrade

DTNB reagent (55' Dithiobis – 2 nitrobenzoic acid)

40 mg of DTNB per 100 ml of 1% sodium citrate solution (1 gm of sodium citrate in 100 ml DW) stored in deep freezer stable for 13 weeks at 4 degree centigrade.

GSH calibrators

100 mg GSH in a 100 ml volumetric flask and bring to volume with reagent grade water. Invert repeatedly until GSH is completely dissolved.

Prepare 50 and 10 mg/dl calibrators.

| Sl no. | Reagents | Test | Blank |
|--------|-----------------|--------|--------|
| 1 | Serum | 1.0 ml | ---- |
| 2 | Distilled water | ----- | 1.0 ml |
| 3 | 40% TCA | 1.0 ml | 1.0 ml |
| 4 | TBA (0.67%) | 2.0 ml | 2.0 ml |

Table 2: Reagents.

| | Reagents | Blank | Assay |
|---|---|-------|-------|
| A | Filterate | -- | 2.0 |
| B | Precipitating reagent | 1.2 | -- |
| C | DW | 0.8 | -- |
| D | NaH ₂ PO ₄ solution | 8.0 | 8.0 |
| E | DTNB solution | 1.0 | 1.0 |

Table 3: Preparation cuvettes.

50 mg/dl : 5 ml of stock solution + 5 ml DW

10 mg/dl : 5 ml of stock solution + 45 ml of DW

GSH calibrator are not stable and must be freshly prepared.

Procedure

1. Place 0.2 ml of whole blood into 10 ml test tube. Add 1.8 ml of DW, mix to hemolyse
2. Promptly add 3.0 ml of precipitating solution, mix.
3. Allow to stand for 5 minute at room temperature and filter through coarse grade filter paper.
4. Prepare cuvettes as follows (Table 3).
5. Cap cuvette and inverts three times to mix.
6. Read absorbance at 412 nm within 4 min of preparing cuvette.
7. Assay GSH calibrators, omitting filtration step.
8. Prepare a calibrator curve. Determine the GSH concentration of whole blood specimen from the graph.
9. Calculate the GSH concentration.

Normal range: 17-39 mg/d

Observations and Discussion

Recent studies have shown that oxidative stress play a vital role in pathophysiology of OCD and symptomatic precipitation during acute attack and even its improvement with supplementation of antioxidants like Vitamin C. The reason why oxidative stress is increased in patients with OCD is still unclear. Patients with OCD do precipitate with symptoms during acute attack of anxiety and stress. Anxiety may cause secretion of catecholamines which in turn may lead to free radical production through NADPH oxidase. Oxidative stress thus developed causes peroxidation of membrane lipids, hence further increasing the symptoms of OCD. Lipid peroxidation releases excess malondialdehyde into blood. Body defense mechanism against free radical injury causes exhaustion of endogenous antioxidants, which depicted in our study with lowered levels of reduced glutathione. From the above facts it is clear that malondialdehyde play a role in pathogenesis of OCD and can be a good marker. So, in the present study an attempt was made to find out the efficacy of malondialdehyde as a marker in patients of obsessive compulsive disorder. We have also compared blood levels of reduced glutathione (GSH) as a marker of antioxidant status in the body.

Free radicals are extremely reactive molecular species with an unpaired electron in its outer most orbit; that persist for only a short period of 10⁻⁹ to 10⁻¹² seconds. Therefore they are difficult to measure directly. Many studies have used an indirect approach to demonstrate free radical reaction with other molecules, such as DNA, lipids or proteins or the levels of antioxidant, according to the hypothesis that their changes are a consequence of the reaction with radicals. When

free radicals overcome the cellular antioxidant defenses oxidative stress takes place potentially leading to damage of all the principal cellular molecules. The brain is especially prone to free radical damage for following reasons:

- It is very rich in polyunsaturated fatty acids, which are particularly vulnerable to free radical induced peroxidation.
- It has low reserve of anti-oxidant enzymes such as glutathione peroxidase & catalase.
- It contains significant amount of iron deposits, despite its low iron binding capacity. Iron ions are known to stimulate free radical generation by virtue of Fenton reaction.

Lipid peroxidation is well established mechanism of cell injury in humans and used as an indicator of oxidative stress in cells and tissues. There is involvement of free radicals and lipid peroxidation in the pathophysiology of OCD. It is suspected that increased level of lipid peroxides, dialdehydes may be due to oxidation of neuronal membrane lipids by free radical. Brain nucleic acids may be metabolized to purine and nucleoside base resulting in excess of adenosine which then becomes substrate for xanthine oxidase pathways that are important in generation of free radicals. Free radicals mediated injury causes peroxidation of lipids present in cell membrane including neuronal membrane, releasing dialdehydes eg. Malondialdehyde. The involvement of free radicals in the pathophysiology of OCD is reflected by rise in the levels of serum malondialdehyde, which is one of the parameters of the study. As a defense mechanism endogenous anti-oxidant reduced glutathione (GSH) clears up the free radicals leading reduced circulating levels of blood GSH.

Results

The collected data was analysed by applying paired t test and coefficient of correlation analysed for the parameters MDA and reduced glutathione levels. P-value less than 0.05 (P<0.05) was considered to be statistically significant (S). P-value less than 0.001 (P<0.001) was considered to be statistically highly significant (HS). P-value more than 0.05 (P>0.05) was considered to be statistically non-significant (NS) (Table 4).

The average age of subjects in controls is 34.03 years and 34.5 years in patients with OCD, which shows that mean age is nearly equal in controls and cases. Sex distribution in controls 17 males and 13 females and in patients with OCD 16 males and 14 females, which shows that sex ratio is nearly equal in controls and cases (Figures 10-14, Tables 5 and 6).

There is significant rise in serum MDA level in OCD cases as compared to the control group (p value is 0.000000653 i.e <0.05). There is significant decrease in blood levels of reduced glutathione (GSH) in OCD cases as compared to control group (p value 0.000000437 i.e <0.05). The Correlation coefficient (CC) between serum MDA levels and blood levels of GSH is - 0.081 (negative). Its 95% Confidence interval is -0.28 to 0.42. Since the value is negative and within the range of confidence interval depicting that parameters MDA and GSH are inversely related i.e extent of rise in MDA levels causes proportional decrease in GSH levels.

| | Cases | Controls |
|-----------------------|-------------|--------------|
| Mean age ± SD (years) | 34.5 ± 9.26 | 34.03 ± 8.52 |
| Sex | 16M/14F | 17M/13F |

Table 4: Baseline characteristics of patients.

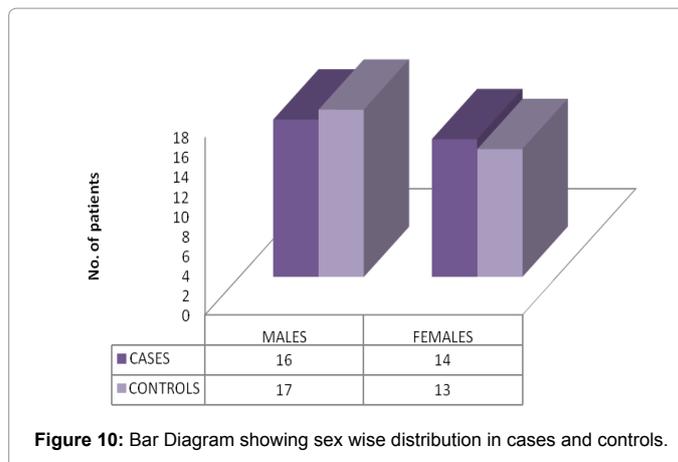


Figure 10: Bar Diagram showing sex wise distribution in cases and controls.

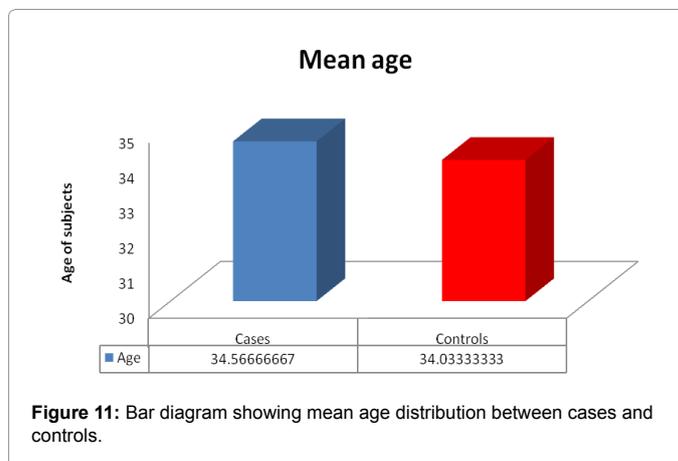


Figure 11: Bar diagram showing mean age distribution between cases and controls.

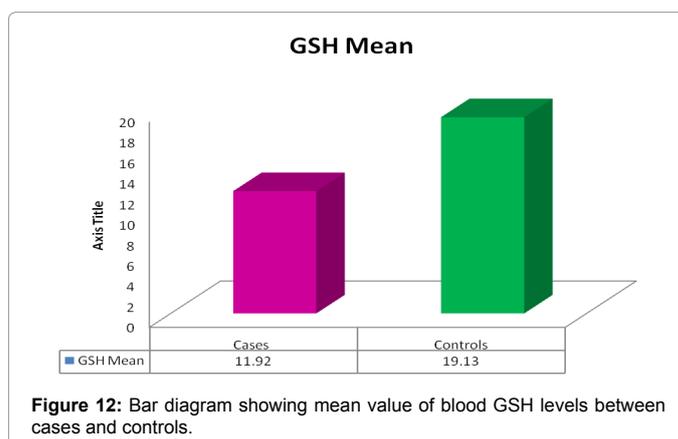


Figure 12: Bar diagram showing mean value of blood GSH levels between cases and controls.

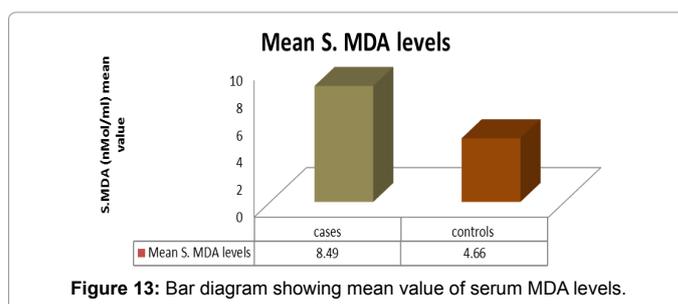
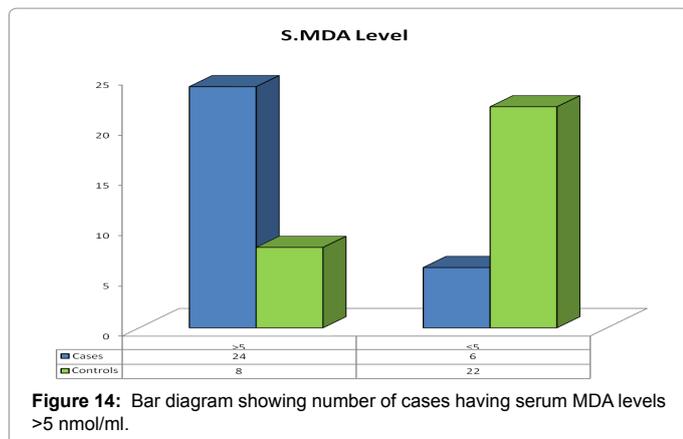


Figure 13: Bar diagram showing mean value of serum MDA levels.



| Group | Blood GSH levels mg/dl Mean | p value |
|----------|-----------------------------|---------|
| Cases | 11.92 ± 6.74 | <0.05 |
| Controls | 19.13 ± 4.17 | |

Table 5: Comparison of blood levels of reduced glutathione (GSH) in OCD cases and controls.

| Group | Serum MDA levels nmol/ml Mean ± SD | p value |
|----------|------------------------------------|---------|
| Cases | 8.49 ± 3.39 | <0.05 |
| Controls | 4.666 ± 1.30 | |

Table 6: Comparison of serum MDA levels in OCD cases and controls.

Hence the present study proves,

- Statistically significant rise in serum MDA levels in cases with respect to controls
- Statistically significant decrease in blood GSH levels in cases with respect to controls
- The existence of negative correlation between MDA and GSH in cases.

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