6

OXIDATIVE STRESS AND INFLAMMATION IN PCOS RAT MODEL

PCOS is a multifaceted disorder associated with hyperinsulinemia and dyslipidemia which pose a risk for the development of metabolic syndromes. The key feature of the metabolic syndromes is the imbalance of redox milieu that leads to increased oxidative stress, further causing inflammation. As PCOS is coupled with symptoms of metabolic syndrome, there could be a plausible association of oxidative stress with the development of PCOS pathology. Thereby, it was thought pertinent to assess the oxidative stress and inflammation related parameters in PCOS.

Oxidative stress

Oxidative stress can be defined as the disturbance of equilibrium between the pro-oxidants and antioxidant molecules that can lead to damage. As the definition suggests, a delicate balance of these moieties is required and any imbalance either due to over-production of pro-oxidants or due to inadequate defence system or both can generate oxidative stress. The pro-oxidant molecules mainly comprise of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are produced in the body through various metabolic processes.

Reactive oxygen species

Reactive oxygen species are unstable metabolites of molecular oxygen which consist of both free radical and non-free radical oxygenated molecules of high reactivity. Free radicals have one or more unpaired electron in the external orbit and when two such kinds of unpaired

electrons fuse, a non-free radical is generated. The unpaired electron in the orbit is responsible for the highly reactive nature of free radicals (Valko et al., 2007). The physiologically important reactive oxygen species are superoxides ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$) and nonradical molecules like hydrogen peroxide (H_2O_2) (Burton & Jauniaux, 2011; Rahal et al., 2014). At low concentrations, these reactive oxygen species play important roles in several normal cellular processes including cell signalling, proliferation and apoptosis (Pisoschi & Pop, 2015; Pruchniak et al., 2015). They are also produced by phagocytes during bacterial or fungal infection and hence serve as an essential component of the natural defence mechanism (Poljsak et al., 2013).

Free radicals are generated in the body through endogenous processes or by exposure to exogenous stimuli. The most common endogenous mechanisms of ROS generation are: i) Oxygen utilized in the mitochondria during aerobic respiration is converted into $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$; ii) H_2O_2 is formed as a by-product of peroxisomal degradation of fatty acids and lipids; iii) phagocytosis of bacterial or virus infected cells stimulates NAD(P)H oxidase which generate nitric oxide (NO), $O_2^{\bullet-}$, H_2O_2 and HClO; iv) Cytochrome P450 family enzymes also produces free radicals during removal dietary and natural toxins (Uttara et al., 2009). Apart from theses, free radicals can also be generated during intensive physical activity or the action of pollutants/toxins such as cigarette smoke, alcohol, ionizing and UV radiations, pesticides, and ozone (Pisoschi & Pop, 2015). Due to their high reactivity, free radicals can directly alter structures of important biomarkers that include lipids, proteins and DNA.

Superoxide anion

The most common free radical is the superoxide anion, a primary ROS that can interact with other molecules to generate secondary ROS (Valko et al., 2005). Superoxides are mainly produced by mitochondrial respiration during which the electron is transferred to different complexes of electron transport chain to generate ATP (Cadenas & Davies, 2000). However, this process is not efficient, leading to leakage of electron from the complexes which then react with oxygen to form superoxide (Burton & Jauniaux, 2011; Pruchniak et al., 2015). Among all the complexes, Complex I and III of the electron transport chain are accountable for most of the superoxide generated in mitochondria. The newly synthesized superoxide anion is highly reactive and can cross the inner mitochondrial membrane to leak out of the mitochondria or it can remain in the mitochondrial matrix (Valko et al., 2007). Similar to

mitochondria, leakage of electrons from shorter electron transport chain of Endoplasmic reticulum can also generate superoxide (Tu & Weissman, 2004). Superoxides can also be generated through various metabolic enzymes like NADPH oxidase, cytochrome P450 and other oxido-reductases (Droge, 2002; Raijmakers et al., 2006).

Hydrogen peroxide

Hydrogen peroxide is mainly synthesized in the peroxisomes under physiological conditions. Peroxisomes participate in several metabolic functions and hence, act as a major site of oxygen consumption which is further converted into H_2O_2 (Valko et al., 2007). Hydrogen peroxide is also generated through several enzymes, which include Catalase, Xanthine oxidase, amono acid oxidase, Monoamine oxidase and Superoxide dismutase (Zhang and Kaufman, 2008; Zhang, 2010; Birben et al., 2012). As H_2O_2 is non-polar, it diffuses through cell membrane and enters into cells where it can act as a secondary messenger for various signal transduction pathways (Burton & Jauniaux, 2011). Also, H_2O_2 can directly bind and oxidize different heme proteins, enzymes, thiol groups, keto-acids, lipids and DNA (Kohen & Nyska, 2002).

Hydroxyl radical

Hydroxyl radical ($HO^{\bullet}$), the neutral form of hydroxide ion is generated through depletion of H_2O_2 by enzymes catalase, glutathione peroxidase and heme containing enzymes. $HO^{\bullet}$ can also be synthesized from H_2O_2 via sequential reactions of Haber-Weiss and Fenton reactions (Birben et al. 2012). $HO^{\bullet}$ is termed as a most potent oxidizing radical species due to its high reactivity with organic and inorganic molecules such as DNA, proteins, lipids, amino acids, sugars and metal ions (Pisoschi & Pop, 2015).

Reactive nitrogen species

Along with oxygen, several nitrogen moieties also act as a free radical, including nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO^{\bullet}_2$) both of which contain unpaired electrons (Gutteridge, 1994). Due to high reactivity, RNS can interact with many molecules and alter their biological activity further leading to nitrosative stress (Ridnour et al., 2004; Valko et al., 2007). Nitric oxide is produced from arginine, oxygen and NADPH in the body through nitric oxide synthase enzymes (NOS) (Valko et al., 2007). $NO^{\bullet}$ is readily soluble in aqueous as well as lipid media and thereby it can easily diffuse through cytoplasm and plasma membranes to affect various processes (Chiueh, 1999; Valko et al., 2007). Further, in presence of superoxide, nitric oxide is converted into the more harmful peroxynitrite radical ($ONOO^-$).

Similar to hydroxyl radicals, reactive nitrogen species can cause protein oxidation and DNA damage (Pisoschi & Pop, 2015).

Increased free radicals due to excessive production or due to diminished antioxidant levels can modulate several classes of biomolecules, ultimately leading to oxidative stress and cellular damage. Lipids are highly susceptible to oxidation by reactive oxygen species which induces lipid peroxidation and produces malonaldehydes and unsaturated aldehydes. These aldehydes can disrupt the membrane lipid bilayer arrangement, increase tissue permeability and can inactivate many enzymes, receptors and proteins via forming cross-linkages (Girotti, 1985; Siu & Draper, 1982; Birben et al., 2012).

Proteins are also damaged by increased reactive oxygen species. Direct oxidation of protein side chain leads to formation of carbonyl groups which are stable and thereby widely used as a marker for protein oxidation (Burton & Jauniaux, 2011). ROS can cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins, and oxidation of specific amino acids and therefore lead to increased susceptibility to proteolysis by degradation via specific proteases (Kelly & Mudway, 2003; Birben et al., 2012). In addition, metal-containing enzymes are also inactivated due to oxidation of their metal ions.

Hydroxyl radicals directly react with DNA bases and deoxyribose sugars leading to DNA strand breakages. Also, oxidation of histone proteins forms cross-linkages that can interfere with chromatin folding, DNA repair and transcription and may result into mutation or altered gene expression (Burton & Jauniaux, 2011). Oxidative damage beyond repair may even activate several caspases, ultimately leading to cell death.

To counterbalance the action of reactive oxygen species, the body is equipped with various antioxidant molecules, which either prevent the generation of reactive oxygen species or convert the already formed radicals into less harmful moieties (Cheeseman & Slater, 1993). The role of each is discussed in following section.

Antioxidants

Antioxidants can be divded into non-enzymatic antioxidants that include Ascorbic acid (Vit C), Tocopherol (Vit E), Glutathione and Carotenoids; and enzymatic antioxidants such as Superoxide dismutase, Catalase, Glutathione peroxidase and Thioredoxin (Birben et al. 2012).

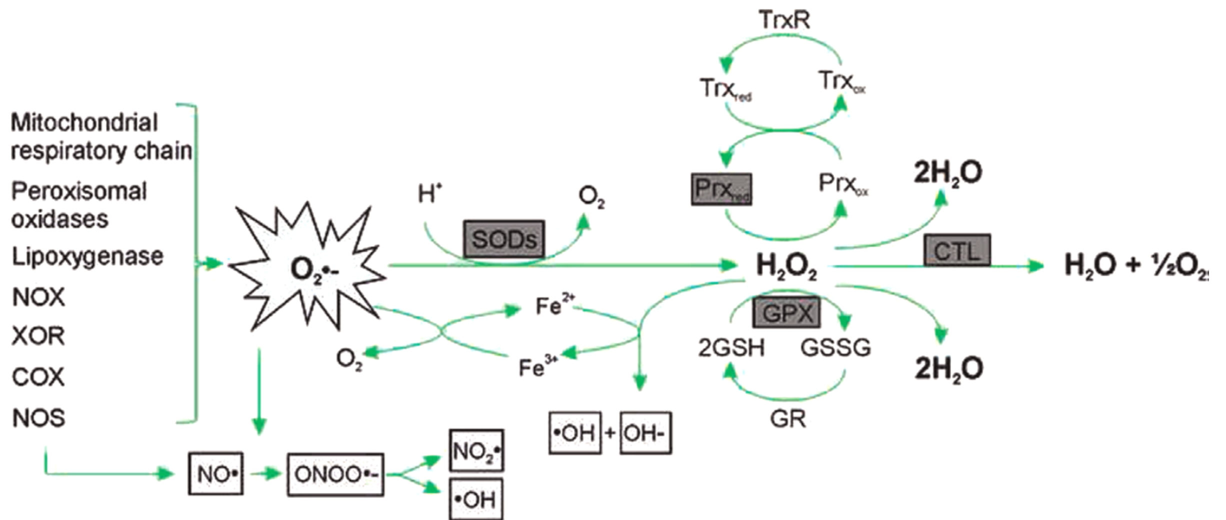


Figure 6.A: Generation of reactive oxygen species and their clearance (Richter et al., 2015)

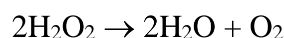
Superoxide Dismutase (SOD)

SOD is a group of metal containing enzymes which play an important role in maintaining the redox status. SOD is the primary enzyme which converts superoxide radical into hydrogen peroxide. There are three different isoforms of SOD in mammalian system: i) SOD1: Copper-Zinc containing SOD (CuZnSOD) which is mainly localized to cytosol; ii) SOD2: Manganese containing SOD (MnSOD) is present in mitochondria and iii) SOD3: (CuZnSOD) which is extracellular. Among all, SOD1 is responsible for majority of cellular dismutation reactions (Pisoschi & Pop 2015).

$$O_2^{\bullet} + 2H^+ \rightarrow H_2O_2$$

Catalase

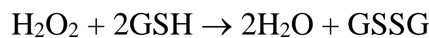
H_2O_2 produced by SOD, Xanthine oxidase, Monoamine oxidase and others oxidase is decomposed to water by the enzymes like Catalase and Glutathione peroxidise. Catalase is a heam containing enzyme which exists as a tetramer of 4 identical monomers (Birben et al. 2012).



$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Glutathione peroxidise (GPx)

Glutathione peroxidise is a selenium-containing, tetrameric enzyme that catalyzes both reduction of H2O2 and organic hydroperoxides to water and alcohol, respectively (Pisoschi & Pop, 2015). GPx utilizes glutathione as a co-substrate to convert H_2O_2 into water. There are mainly four isoforms of GPx: i) GPx1 is the most ubiquitously found cellular form; ii) GPx2 is mainly found in the gastrointestinal tract; iii) GPx3 is present in extracellular compartment; iv) GPx4 is membrane bound and mostly involved in reduction of esterified lipids (Birben et al., 2012).



$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

Glutathione (GSH)

GSH, a tripeptide (γ-L-Glutamyl-L-cysteinylglycine), is the most abundantly found antioxidant in cellular compartments that can reduce H_2O_2 and peroxylipids by the enzyme GPx and Glutathione transferase respectively. Along with this, it can also act as a non-enzymatic antioxidant by directly scavenging hydroxyl anions and chlorinated oxidants (Sung et al., 2013). It can also prevent formation of peroxynitrite by converting nitric oxide into *S*-nitrosoglutathione, a less harmful product (Birben et al., 2012).

Oxidative stress and inflammation are interrelated and a large body of evidence suggests that inflammation induces production of reactive oxygen species, while oxidative stress promotes and aggravates inflammation, forming a vicious cycle (Hulsmans & Holvoet, 2010; Duleba & Dokras, 2012).

Inflammation

Inflammation can be defined as the body's defence system against harmful stimuli such as pathogens and cellular damage by intervention through cytokines, immune cells and inflammatory mediators. Inflammation can be of two types – acute and chronic. Acute inflammation is an initial stage, mainly beneficial to the body and mediated by immune cells. Chronic inflammation may result from a longer exposure to inflammatory stimuli and it may predispose to several illness (Lin & Karin, 2007; Reuter et al., 2010).

Inflammation induces cytokines, chemokines and arachidonic acid metabolites which can activate signal transduction of several transcription factors such as Nuclear factor kappa B (NFκB), Signal transducer and activator of transcription 3 (STAT3), Hypoxia-inducible factor-1α (HIF1α), Activator protein-1 (AP1), etc. and mediate immediate cellular stress

response (Reuter et al., 2010). Furthermore, oxidative stress-induced inflammation also involves activation of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and increases expression of inflammatory cytokines – Tumor necrosis factor (TNF), interleukin-1 (IL1), IL6 and chemokines (Hussain & Harris, 2007). Also, during inflammation, mast cells and leukocytes increase oxygen uptake and thereby increases generation of reactive oxygen species (Coussens & Werb, 2002; Hussain et al., 2003). This increased oxidative stress and inflammation can lead to a vicious cycle, which can damage normal cellular function and result into disease conditions (Reuter et al., 2011). Emerging data suggests an association of inflammation with hyperglycemia, dyslipidemia and hyperinsulinemia, leading to the development of metabolic syndrome (Shoelson et al., 2006; Ando & Fujita, 2009; Monteiro & Azevedo, 2010). Also, recent reports indicate the role of sustained inflammatory and oxidative environment in several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease as well as in psychological disorders including depression (Uttara et al. 2009; Kato et al. 2013; Oakes et al., 2017). Among all, the association of inflammation with depression will be discussed further.

Depression

Depression is the most common psychiatric disorder which is associated with persistent sadness, anxiety, helplessness, low self-esteem and aversion of activity.

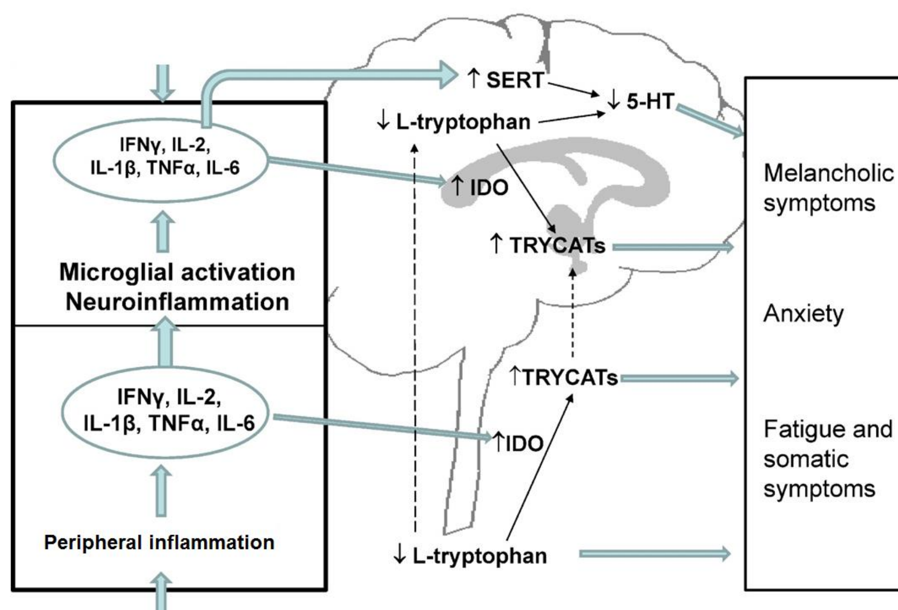


Figure 6.B: Inflammation and depression (Leonard & Maes 2012)

It has long been known as the disorder of psychological origin but recent reports suggest the role of biochemical and cellular alterations in depression pathology (Young et al., 2014; Oakes et al., 2016; Bakusic et al., 2017). Also, emerging data suggest the involvement of inflammation and oxidative stress in pathogenesis of depression. Increased inflammation results into increased production of IL1β, TNFα and IL6 through activation of cell-mediated immunity, which increases IFNγ and IL2. These inflammatory molecules decrease serotonin levels in the synapse by either increasing its enzymatic degradation through indoleamine-2,3-dioxygenase (IDO) or by increasing serotonin reuptake by serotonin reuptake transporter (SERT), which would result into depression-like symptoms (Leonard & Maes, 2012).

Reports also indicate that increased oxidative stress and inflammation decrease the expression of brain-derived neurotrophic factor (BNDF) and its receptor tyrosine kinase 2 (TrkB) (Miller et al., 2009). BDNF/TrkB is an essential component for neuronal cell growth, differentiation and survival and its reduction increases the susceptibility to damage by reactive oxygen species and inflammation and may result in cell death (Acheson et al., 1995; Huang & Reichardt, 2001). Patients with unipolar and bipolar depression have shown significant low levels of BDNF compared to healthy controls, suggesting a role for BDNF as an important neuroprotectant (Miller et al., 2009).

All the above-cited literature indicates that proper cell functioning requires a balance between reactive molecules such as ROS, RNS, inflammation and the body's defence mechanism to eliminate such moieties. Any disturbance in this delicate network may exert cellular damage and thereby lead to disease pathology.

Rationale

Polycystic ovarian syndrome is a widespread endocrine disorder and recent reports suggest the role of oxidative stress and inflammation as one of the etiological factors for PCOS (Escobar-Morreale et al., 2011; Gonzalez, 2012). In fact, polycystic ovarian syndrome is known for its pro-inflammatory state and emerging data implies that chronic low-grade inflammation underpins the development of metabolic aberration and ovarian dysfunction in the disorder (Gonzalez, 2012; Shorakae et al., 2015). Also, PCOS is a pathology wherein hyperinsulinemia is implicated which may lead to increased generation of advanced glycation end products (AGEs) that in turn may precipitate as inflammation (Diamanti-Kandarakis, 2008). In this line, reports indicate increased inflammatory markers such as IL-1β, IL-6,

TNF-α and IFN-γ, among other markers in serum of PCOS women (Gonzalez et al., 1999; Amato et al. 2003; Deligeoroglou et al., 2012). Furthermore, proinflammatory cytokines can directly increase ROS production and also affect negatively the oxidative environment (Hulsmans & Holvoet, 2010; Duleba & Dokras, 2012). In this line, reports suggest that oxidative stress is associated with PCOS. The serum of women suffering with PCOS has been shown to have an altered antioxidant profile (Murri et al., 2013b; Piomboni et al., 2014). Also, women with PCOS have shown increased blood levels of malondialdehyde (MDA), a product of lipid peroxidation and decreased levels of reduced glutathione content when compared with BMI and age matched controls (Sabuncu et al., 2001; Murri et al., 2013b).

With reference to above literature, it is clear that PCOS has been linked with increased oxidative stress and inflammation. However, majority of the studies have been performed using blood, follicular fluid and oocytes isolated from PCOS women. As demonstrated in the previous chapters, PCOS is associated with defects in several organ systems including brain and adrenal. Also, PCOS rats demonstrated hyperandrogenic and hypoestrogenic condition (Chapter 3). It is known that estrogen acts as an antioxidant and low levels of estrogen poses a risk of oxidative stress development (Bellanti et al., 2013; Unfer et al., 2015). As PCOS is a metabolic syndrome associated with hyperinsulinemia, hyperglycemia and increased HOMA-IR index (Chapter 3), the susceptibility for inflammation and oxidative stress occurrence is high. Furthermore, increased stress and glucocorticoids are linked with oxidative damage and mitochondrial dysfunction (Abidin et al., 2004; Zhang et al., 2006) and present study has demonstrated adrenal hyperactivation with increased corticosterone in PCOS (Chapter 4). In addition, results have demonstrated elevated levels of Glutamate in brain regions of PCOS rats with increased MAO activity (Chapter 5). In this context, a report indicates that increased glutamate can lead to excitotoxicity and cellular damage (Nguyen et al., 2011). Also, increased activity of MAO indicates heightened production of H_2O_2 in PCOS. Taken together, the microenvironment of the brain is more prone to oxidative stress in PCOS condition. In the same line, studies have demonstrated the presence of systemic as well as ovarian oxidative stress in PCOS (Sabuncu et al., 2001; Duleba & Dokras, 2012; Murri et al., 2013; Lin et al., 2014; Seleem et al., 2014). However, the redox status of the GnRH regulatory brain regions has not been studied in PCOS condition. Thereby, an aim of the present study was to demonstrate the status of antioxidants and its implication in PCOS pathology.

Material and Methods

Letrozole-induced PCOS model was developed as described earlier in materials and methods (Chapter 2). For analysis of oxidative stress parameters, various antioxidant enzymes were assayed for their activities in the blood as well as tissues such as hypothalamus, pituitary, hippocampus, frontal cortex, ovary and adrenal. For estimation of superoxide dismutase and catalase, kinetic method was employed, whereas glutathione peroxidise activity and reduced glutathione content was measured spectrophotometrically. Furthermore, mRNA expression of antioxidant enzymes *SOD1*, *CAT* and *GPX1* were performed using real-time PCR. Nitric oxide amount was measured indirectly using a Griess reagent-based method. For evaluation of cellular damage, lipid peroxidation and protein carbonylation was estimated. For DNA damage analysis, western blot of Caspase-3 protein was carried out. Inflammatory markers such as TNFα (*TNFα*), IFNγ (*IFNγ*), IL1β (*IL1B*), IL2 (*IL2*), IL6 (*IL6*) and IL-10 (*IL10*) as well as inflammation sensitive genes SERT (*SERT*), IDO (*IDO1*), BDNF (*BDNF*) and TrkB (*TrkB*) were evaluated by real-time PCR. In addition, for behavioural analysis, several tests were employed. Food and water intake was measured throughout the course of the letrozole treatment. For the assessment of depression-like behaviour, forced-swim test, tail-suspension test, open field test and sucrose preference tests were performed. These have been described in Chapter 2.

Results

Oxidative stress in PCOS

PCOS rats demonstrated a significant reduction in the activity of Superoxide dismutase (SOD) ($P<0.05$), Catalase ($P<0.01$) and Glutathione peroxidase (GPx) ($P<0.01$) with decreased content of glutathione ($P<0.001$) in blood as compared to control animals (Figure 6.1). On the other hand, nitric oxide (NO) levels were markedly elevated in PCOS animals ($P<0.001$) as compared to control rats, thereby indicating that PCOS is associated with increased oxidative and nitrosative stress. To further check the incidence of oxidative stress in different tissues of PCOS, oxidative parameters were measured in brain regions (Figure 6.2 and 6.3), ovary and adrenal gland (Figure 6.3). Hypothalamus of PCOS rat demonstrated notable reduction in the activity of antioxidant enzymes SOD ($P<0.05$), Catalase ($P<0.05$) and GPx ($P<0.01$) with no change in the mRNA transcripts of *SOD1*, *CAT*, *GPX1* when compared with control animals (Figure 6.2). Also, GSH levels were significantly reduced ($P<0.001$) while marked increase was observed in NO ($P<0.001$) content in the hypothalamus

of PCOS animals as compared to control group. When analysed for the oxidative stress parameters in pituitary gland (Figure 6.2), similar pattern of changes was observed as seen in case of hypothalamus, with a more profound decrease in SOD (P<0.001) activity and reduced *SOD1* (P<0.01) expression as compared to control pituitary gland. No significant difference was observed in activity or mRNA transcripts of SOD, Catalase and GPx in hippocampus of PCOS and control animals (Figure 6.2). However, hippocampal GSH was reduced (P<0.05) with increased NO levels (P<0.05) in PCOS animals as compared to control animals. Oxidative stress parameters in frontal cortex (Figure 6.3) were unaltered except for a marked decrease in GPx activity (P<0.05) with low levels of GSH (P<0.05) in PCOS animals as compared to control animals. A significant decrease in SOD activity (P<0.01) and GSH levels (P<0.01) and notable increase in NO content (P<0.001) was observed in ovary of PCOS animals with no change in Catalase, GPx activity and *SOD1*, *CAT*, *GPX1* mRNA transcript. Adrenal gland of PCOS animals demonstrated a significant reduction in GPx activity (P<0.01), GSH content (P<0.01) and *GPX1* mRNA expression (P<0.01) whereas NO levels were markedly high (P<0.001) as compared to control adrenal glands (Figure 6.3).

Cellular damage

Increased oxidative stress can damage important cellular components such as lipids, proteins and DNA. Thereby, lipid peroxidation (LPO), protein carbonylation and Caspase-3 activation was measured in PCOS condition (Figures 6.4 and 6.5). Hypothalamus of PCOS rats demonstrated significantly increased lipid peroxidation (P<0.05), protein carbonylation (P<0.05) and cleaved Caspase-3 expression (P<0.05) as compared to control animals. Similarly, increased LPO levels (P<0.01), protein carbonylation (P<0.01) and Caspase-3 activation (P<0.05) was observed in pituitary of PCOS animals as compared to control group. There was no significant difference was observed for LPO, protein carbonylation and Caspase-3 expression in hippocampus and frontal cortex of the control and PCOS group. When analyzed for these parameters in ovary, significant increase in LPO (P<0.01), protein carbonylation (P<0.05) and cleaved Caspase-3 expression (P<0.05) was observed in PCOS animals. Protein carbonyl groups were notably elevated in adrenal gland of PCOS animals (P<0.05) whereas no change was observed in lipid peroxidation and Caspase-3 expression as compared to control adrenal tissue.

Inflammation

Oxidative stress and inflammation are interconnected and disruption in one may alter another. Significant modulations in oxidative markers were observed in PCOS condition. Thereby, status of inflammatory markers was evaluated by real-time PCR in PCOS animals (Figures 6.6 and 6.7). The transcripts of pro-inflammatory genes *TNFα* ($P<0.001$), *IFNγ* ($P<0.01$), *IL1B* ($P<0.05$), *IL2* ($P<0.01$) and *IL6* ($P<0.01$) were significantly increased whereas anti-inflammatory gene *IL10* ($P<0.01$) was significantly reduced in hypothalamus of PCOS animals as compared to control group. Pituitary gland of PCOS animals also followed similar pattern of change in inflammatory markers as observed in hypothalamus. Expression of *TNF* ($P<0.01$), *IL2* ($P<0.05$) and *IL6* ($P<0.05$) was markedly elevated while that of *IL10* ($P<0.05$) was notably reduced in hippocampus of PCOS animals as compared to control rats. Frontal cortex of PCOS animals exhibited a prominent increase in *IFNγ* ($P<0.05$) and *IL2* ($P<0.05$) mRNA expression while expression of other inflammatory genes was unaltered. PCOS animals demonstrated significant increase in the ovarian transcripts of *TNF* ($P<0.05$), *IFNγ* ($P<0.01$), *IL1B* ($P<0.01$) and *IL2* ($P<0.01$), while no change was observed in *Il6* and *Il10* expression as compared to control animals. Gene expression of adrenal gland revealed marked elevation of *TNFα* ($P<0.05$), *IFNγ* ($P<0.01$) and *IL2* ($P<0.05$) transcripts in PCOS animals while expression of *IL1B*, *IL6* and *IL10* remain unchanged. Current study demonstrated an increase in the pro-inflammatory cytokine in the PCOS condition which could result into increased inflammation.

Several neuronal regulators like SERT, IDO, BDNF and TrkB are highly sensitive to inflammation and any change in inflammatory markers can exert deleterious effect in neural processes through altering these molecules. When analysed for gene expression (Figures 6.8 and 6.9) both hypothalamus and pituitary demonstrated significant increase in the transcripts of *SERT* ($P<0.01$) and *IDO1* (IDO) ($P<0.001$) while the transcripts of *BDNF* (BDNF) and *TrkB* (TrkB) were significantly reduced ($P<0.01$) in PCOS animals as compared to control animals. Similar trend of expression was also observed in hippocampus and frontal cortex of PCOS animals wherein *SERT* ($P<0.05$) and *IDO1* (IDO) ($P<0.05$) transcripts were elevated while *BDNF* ($P<0.05$) and *TrkB* ($P<0.05$) expression decreased in comparison to control animals. When analyzed for ovarian transcripts, *IDO1* (IDO) ($P<0.05$) was significantly increased whereas *BDNF* ($P<0.01$) and *TrkB* ($P<0.01$) were markedly reduced in PCOS animals. There was no significant difference found for transcripts of adrenal tissue between

control and PCOS group. However, expression of *BDNF* ($P<0.01$) was notably high in adrenal of PCOS rats as compared control animals.

Behaviour analysis

Increased oxidative stress and inflammation interferes with several processes in brain which could precipitate into behavioural anomalies. Thereby, several behaviour tests were performed in PCOS condition (Figure 6.10). There was no significant difference observed in the food and water intake during the course of letrozole treatment between the two groups. In forced-swim test (FST) and tail-suspension test (TST), control animals were vigorously moving to escape from the unfavourable/threatening condition. In contrast, PCOS animals were immobile for significant amounts of time during FST ($P<0.01$) and TST ($P<0.001$) as compared to control animals. Similarly, locomotor activity was reduced with decreased grooming behaviour in PCOS animals compared to control animals during open field test ($P<0.001$). Also, in sucrose preference test, control animals consumed more amount of sucrose solution whereas PCOS animals preferred water instead of sucrose solution ($P<0.001$). Above-stated results indicate that PCOS animals exhibit a depression-like state.

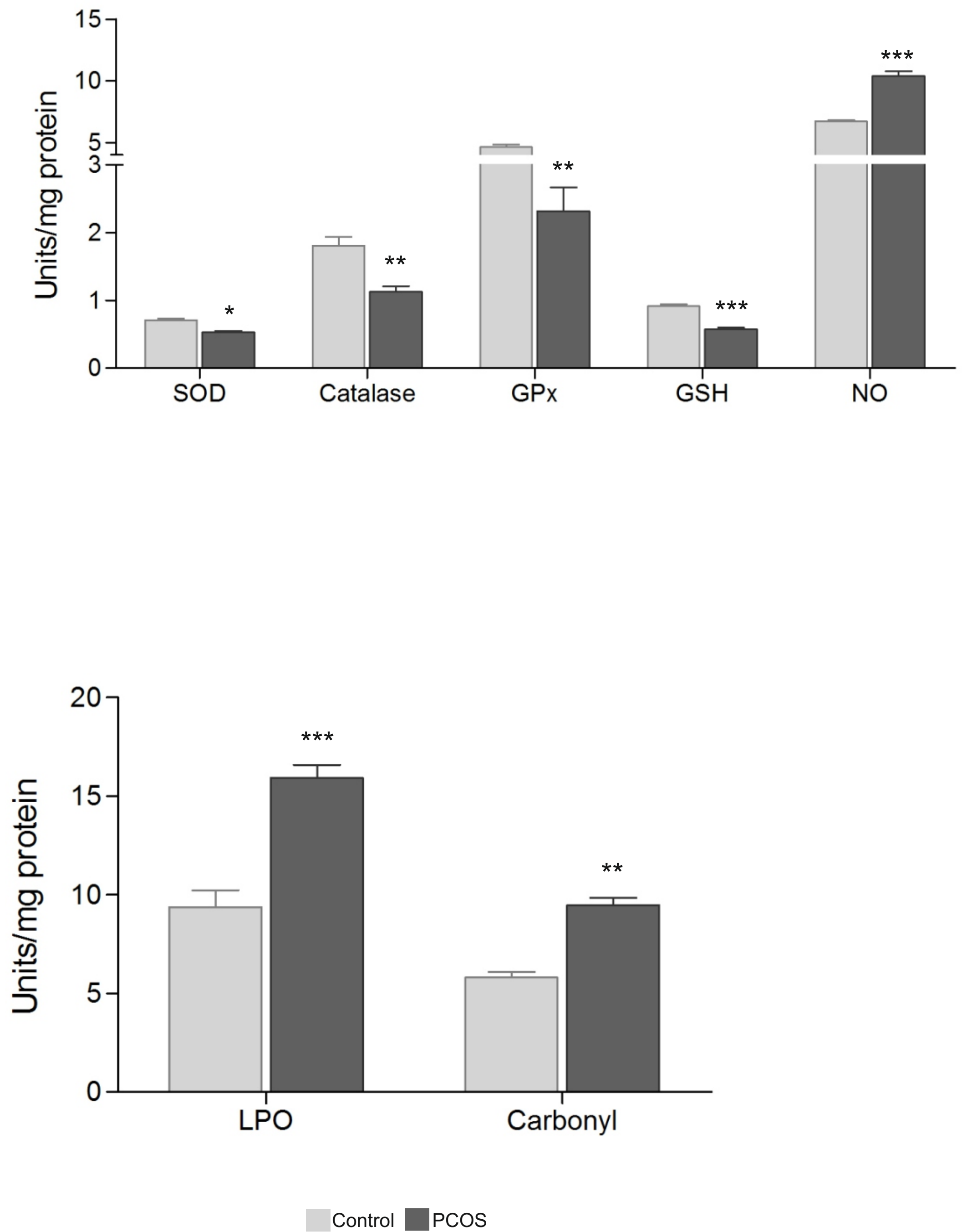


Figure 6.1: Levels of oxidative stress markers in serum of control and PCOS group animals. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

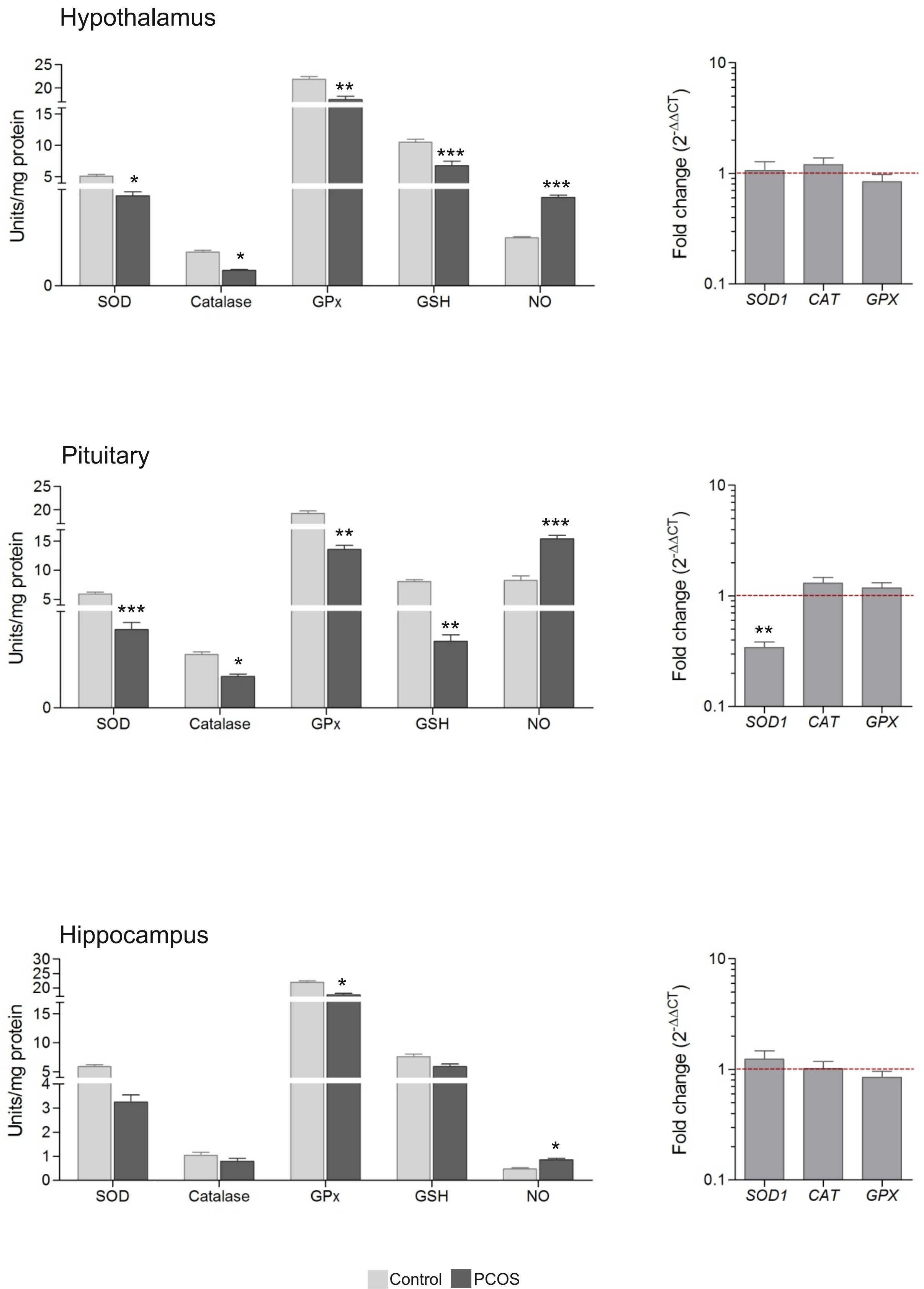


Figure 6.2: Levels of oxidative stress markers in control and PCOS group animals (Left). Relative gene expression of oxidative stress markers (Right). Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to control group.

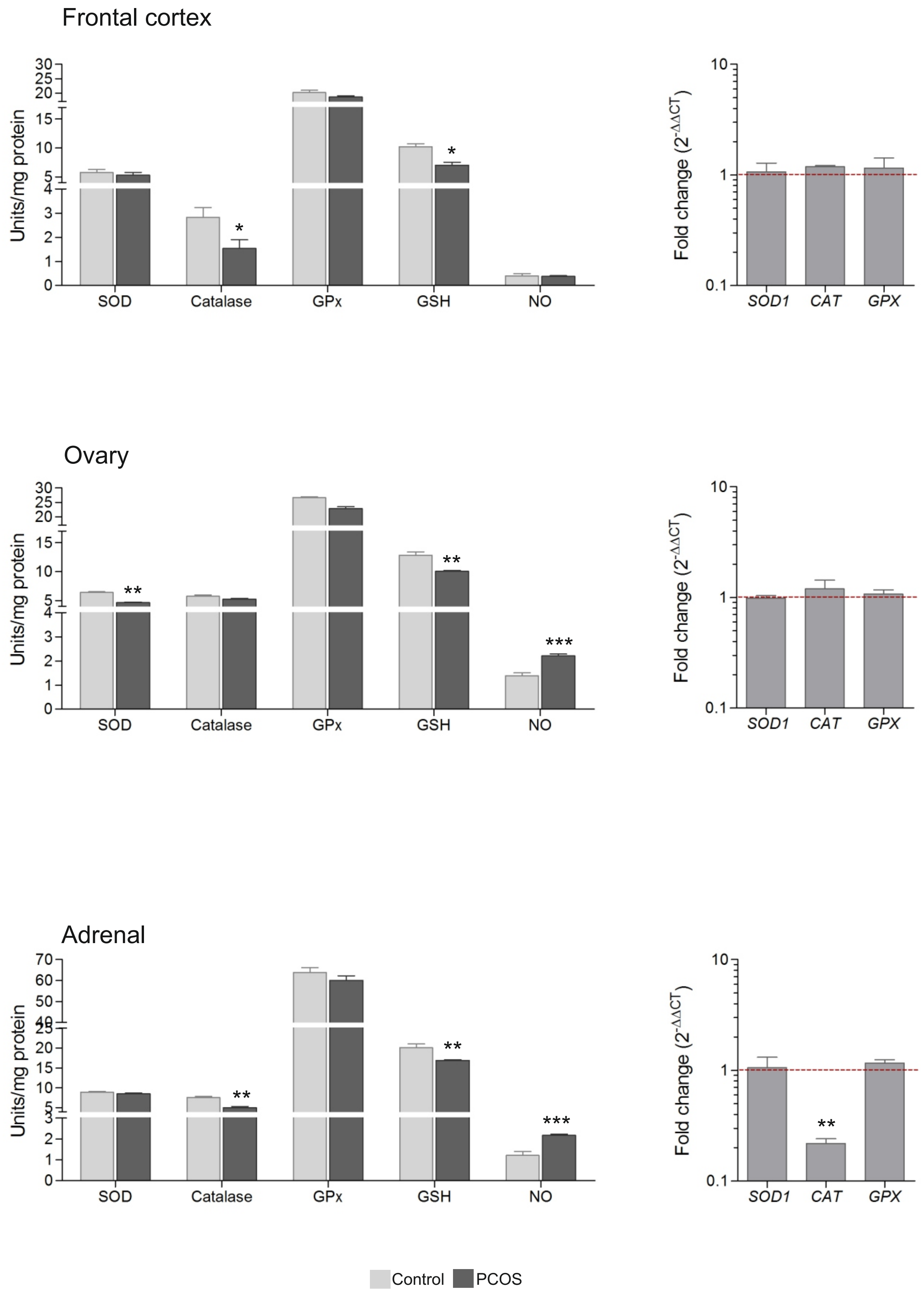


Figure 6.3: Levels of oxidative stress markers in control and PCOS group animals (Left). Relative gene expression of oxidative stress markers (Right). Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

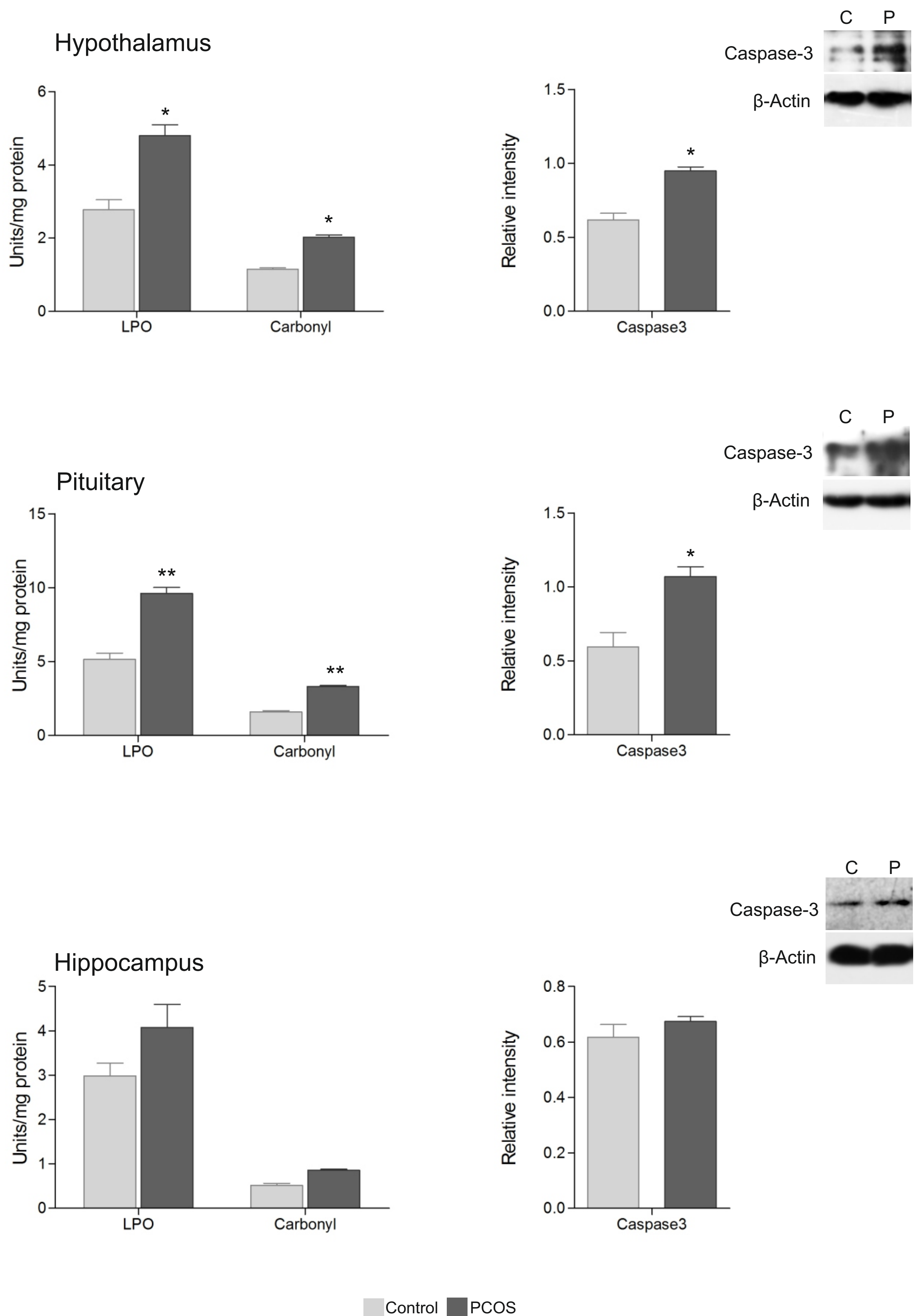


Figure 6.4: Cell damage parameters in control and PCOS group animals. Left: Lipid peroxidation and protein carbonylation level. Right: Relative band intensities for Caspase-3 immunoblot. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

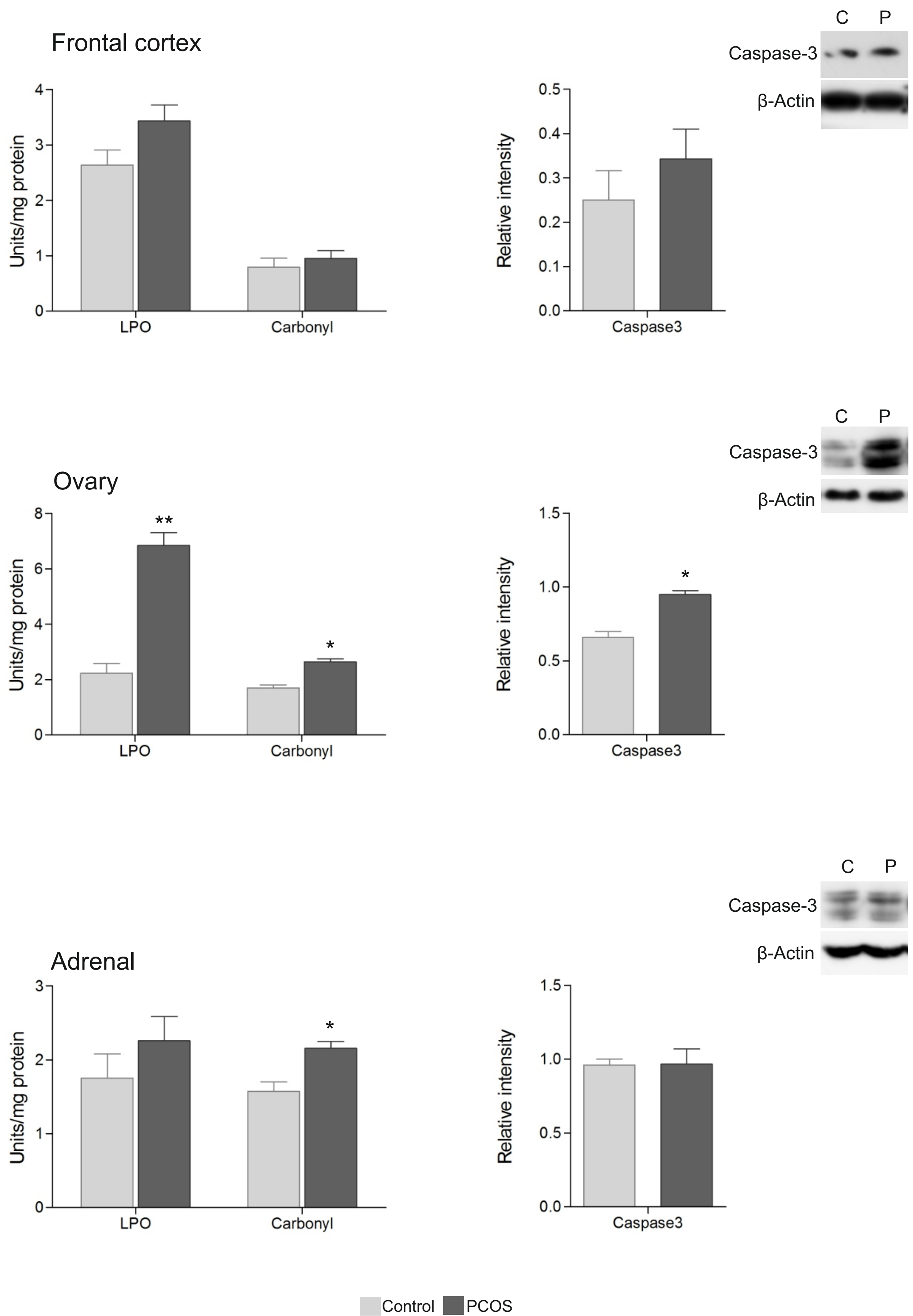


Figure 6.5: Cell damage parameters in control and PCOS group animals. Left: Lipid peroxidation and protein carbonylation level. Right: Relative band intensities for Caspase-3 immunoblot. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

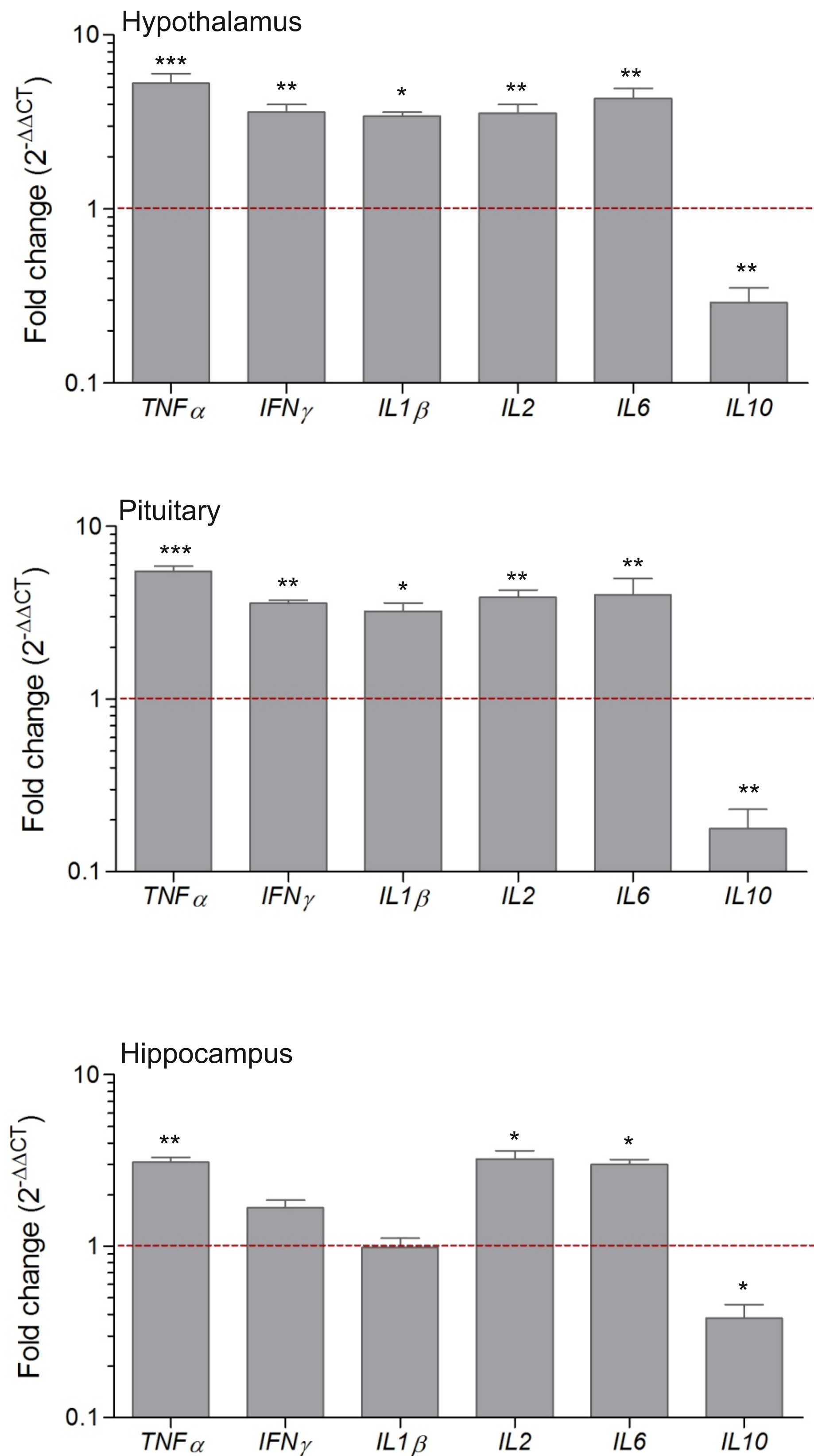


Figure 6.6: Relative gene expression of inflammatory markers. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

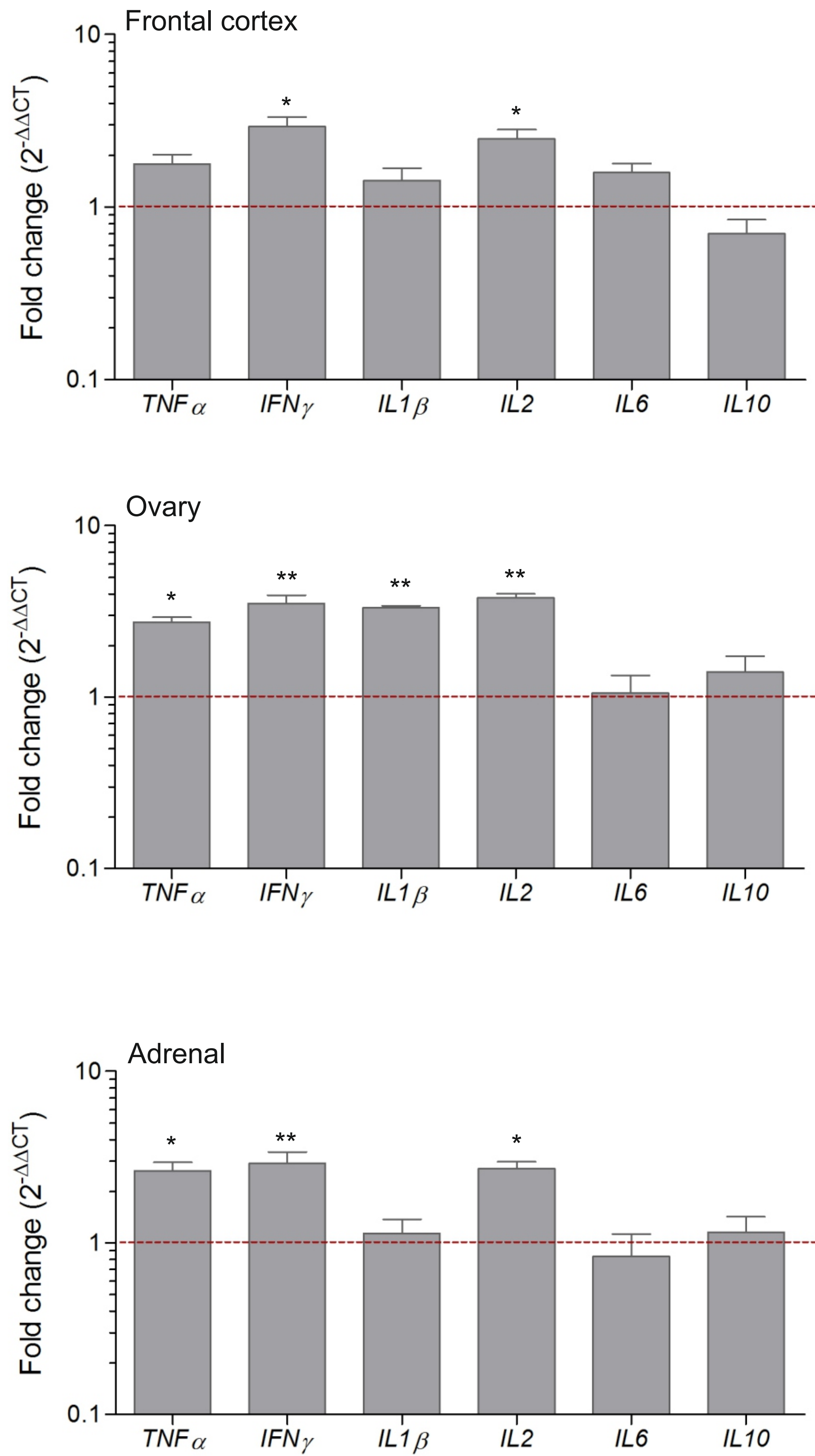


Figure 6.7: Relative gene expression of inflammatory markers. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

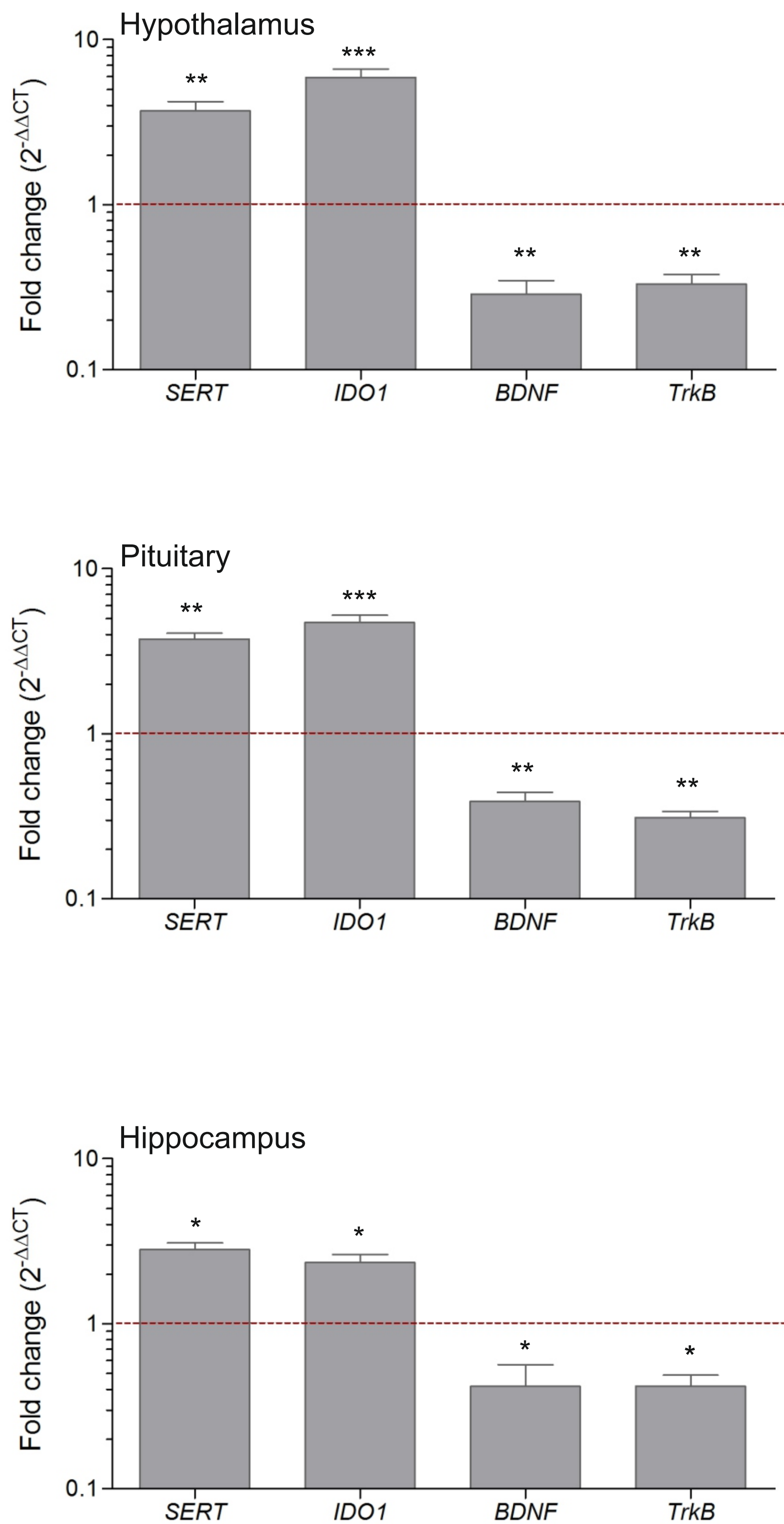


Figure 6.8: Relative expression of inflammation-sensitive genes. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

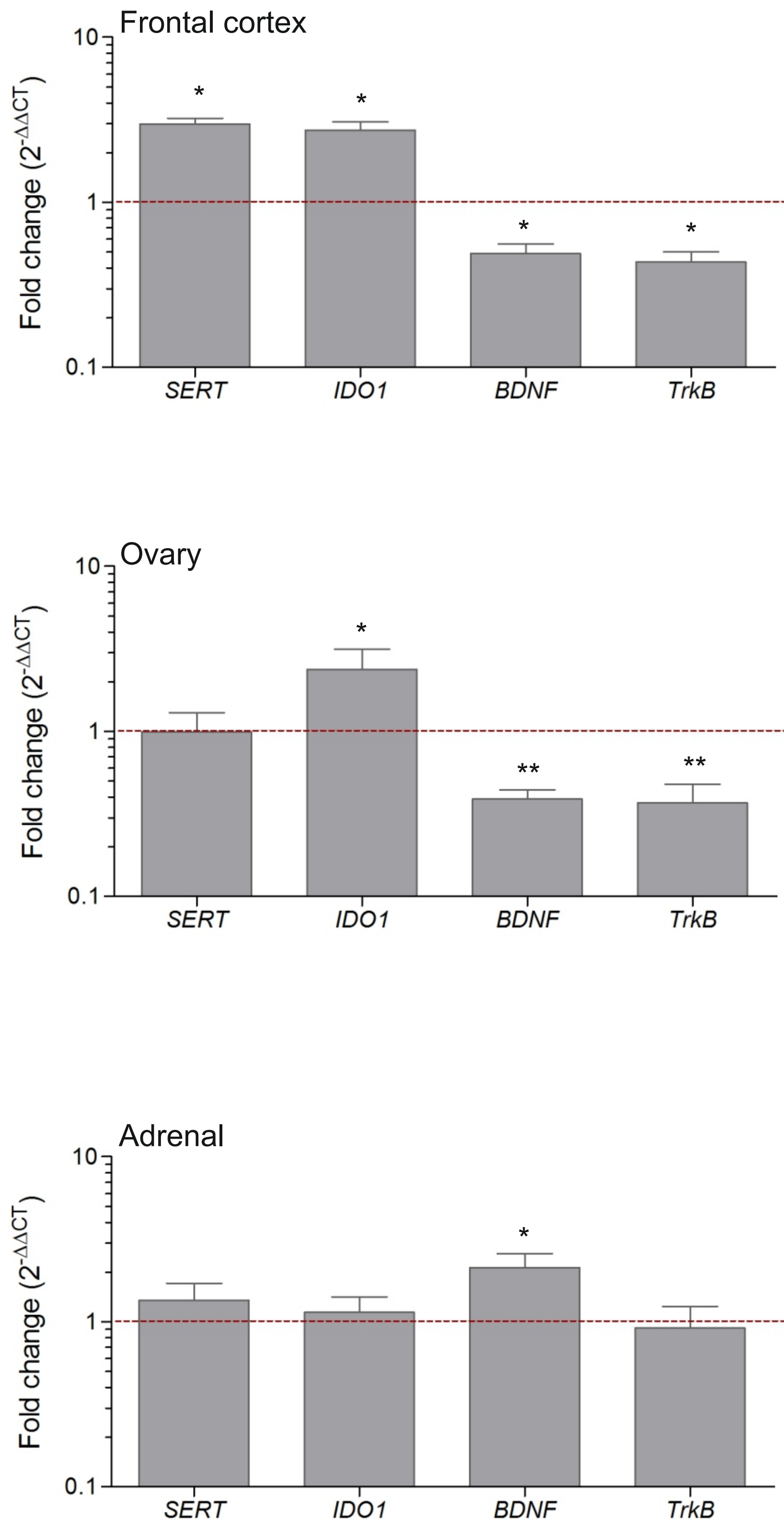


Figure 6.9: Relative expression of inflammation-sensitive genes. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

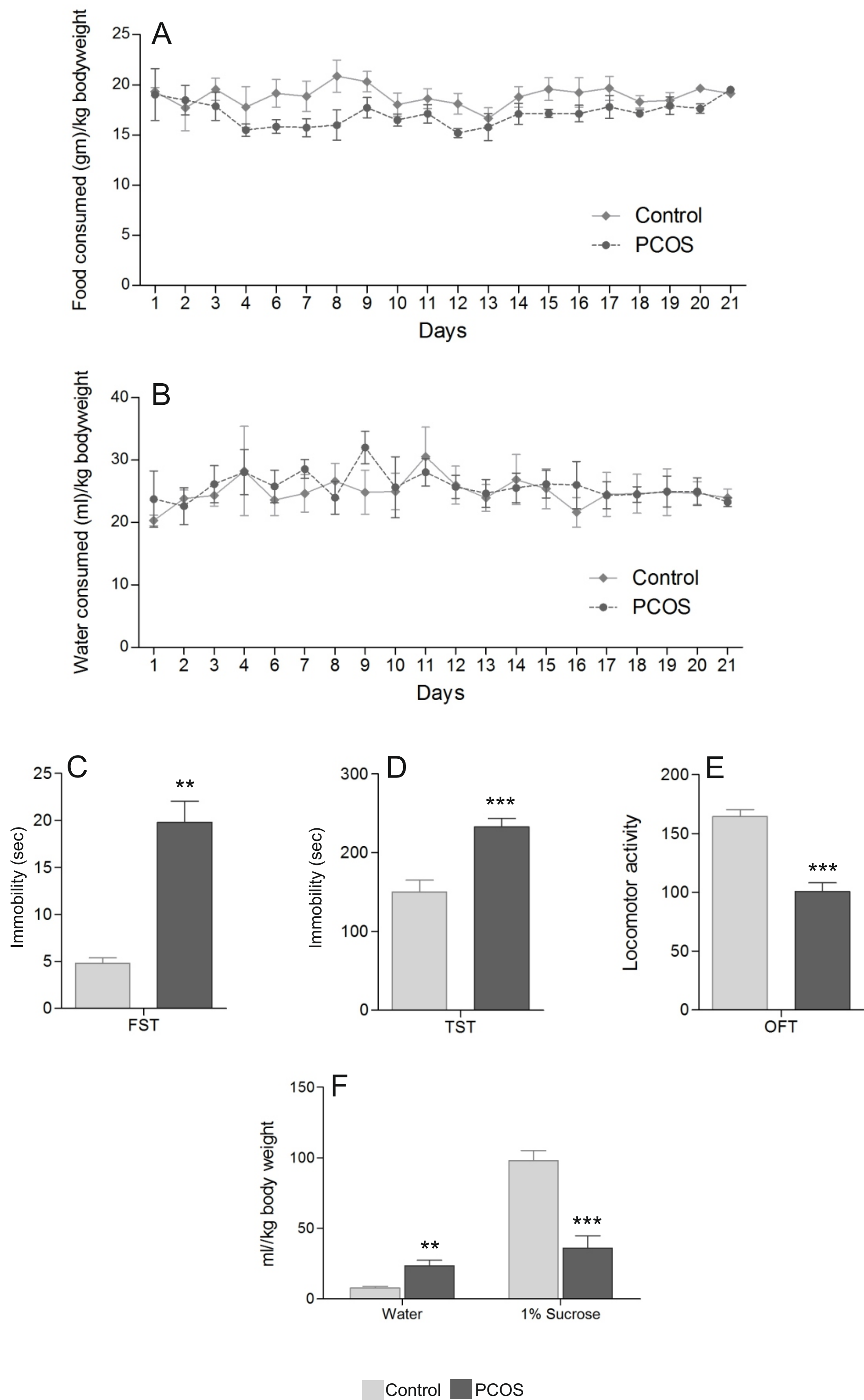


Figure 6.10: Behavioural test results in control and PCOS animals. Quantity of food consumed during the course of treatment (A); Quantity of water consumed during the course of treatment (B); Time spent immobile in the forced-swim test (C) and in the tail suspension test (D); Locomotor activity as seen in the open field test (E); Results of sucrose-preference test. Error bars represent SEM; N=10 per group. *P<0.05; **P<0.01; ***P<0.001

Discussion

PCOS is the most common endocrine disorder, affecting women in their reproductive age. Although it is a reproductive disorder, presence of many metabolic aberrations including dyslipidemia, hyperinsulinemia, and obesity are common features of PCOS which may further culminate into metabolic syndrome (Teede et al., 2010). The most common feature of metabolic syndrome includes the elevation of oxidative stress and inflammatory markers which underpins many of the associated conditions (Ando & Fujita, 2009; Cai & Liu, 2012). In this context, reports have indicated increased oxidative stress and inflammation in women with PCOS. PCOS women have increased serum levels of proinflammatory cytokines TNFα, IL1β and IL6 in comparison to healthy women (Gonzalez et al., 1999; Amato et al., 2003; Deligeoroglou et al., 2012). Also, MDA, a product of lipid peroxidation was elevated while the level of antioxidant glutathione was significantly reduced in women with PCOS when compared with age- and BMI-matched controls (Sabuncu et al., 2001; Murri et al., 2013). Similar to this, the present study has demonstrated a significant degree of increase in oxidative stress markers which was reflected by decreased activity of serum antioxidant enzymes SOD, Catalase and GPx and reduced levels of glutathione. Also, lipid peroxidation and protein carbonylation were both markedly elevated; indicating increased oxidative stress in PCOS animals.

The increased oxidative stress and inflammation in PCOS is linked with visceral adiposity, hyperglycemia and hyperandrogenemia (Teede et al., 2010; Duleba & Dokras, 2012; Gonzalez, 2013; Zuo et al., 2015). Visceral adipocytes are known to secrete several molecules including pro-inflammatory cytokines and these molecules, through endocrine or paracrine action, affect various body functions (Duleba & Dokras, 2012). Increased visceral adiposity has been observed in all women with PCOS, irrespective of their age, body weight and BMI, indicating the over production of pro-inflammatory cytokines in PCOS (Carmina et al., 2007; Duleba & Dokras, 2012). Furthermore, increased production of TNFα and IL1 interferes with insulin signaling by incresaing serine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to insulin resistance (Shoelson et al., 2006). Similar changes may be accounted in our study wherein hyperglycemia persists, which may result into altered insulin signaling.

The role of hyperandogenemia and hyperglycemia in predisposition of inflammation and oxidative stress has been found to be bidirectional (Gonzalez et al., 2010). In contrast to

monocytes of normally ovulating women, monocytes of PCOS women are highly sensitive to glucose uptake (Gonzalez et al., 2005; 2006). The pre-activated monocytes in PCOS women produce reactive oxygen species and they activate NFκB, further increasing the transcription of TNFα and other proinflammatory cytokines (Gonzalez et al., 2007). Furthermore, acute administration of androgen stimulates ROS generation and activates NFκB and TNFα mRNA from monocytes of normal ovulatory women upon glucose ingestion (Gonzalez et al., 2010). This indicates that hyperandrogenemia is a progenitor of diet-induced inflammation in PCOS (Gonzalez, 2013). The increased oxidative stresss and inflammatory markers observed in the present study can be well correlated with hyperandrogenic and hyperglycemic condition (Chapter 3) found in PCOS. Furthermore, increased ROS can lead to cross-linking of reducing sugars with amino groups of proteins, resulting into highly reactive advanced glycation end products (AGEs), which further increases oxidative stress (Duleba & Dokras, 2012). Usually, high levels of AGEs are observed in diabetic pateints but recent studies have also demonstrated elevation of AGEs in PCOS women (Diamanti-Kandarakis et al., 2008; 2009b), indicating that insulin and hyperglycemia also contribute to increased oxidative stress in PCOS. The results of present study are simultaneous with PCOS women wherein increased oxidative stress, inflammation, hyperandrogenemia, hypergylcemia and adipocyte dysfunction has been observed. However, the crosstalk of these molecules is complex and whether it is a cause or consequence of PCOS is still an enigma.

In addition to systemic level elevation of oxidative stress and inflammation, studies have also demonstrated reduced activity of SOD and GPx in follicular fluid isolated from PCOS women (Lin et al., 2014; Seleem et al., 2014). Also, granulosa cells of PCOS patients have low levels of GSH and higher lipid peroxidation (Lin et al., 2014). Similar results were also observed in the present study with letrozole-induced PCOS model. Increased expression of activated monocytes was demonstrated in PCOS ovary, indicating increased release of pro-inflammatory cytokines in the ovary (Best et al., 1996). Increased inflammatory stimuli may also increase ovarian steroidogenesis by increasing the expression of Cyp17 as shown by a study *in vitro*. Further, the action on steroidogenesis was reverted when cells were incubated with anti-inflammatory agents (Ortega et al., 2011; Piotrowski et al., 2005). In addition, TNFα is also able to increase proliferation of androgen-producing theca cells (Spazynsky et al., 1999). All these reports indicate that increased oxidative stress and inflammation may lead to increased androgen prodction in a polycystic ovary. Although oxidative stress is important for normal follicular development, evidence also suggests that increased oxidative

stress may lead to deleterious effects on folliculogenesis (Agarwal et al., 2005). Increased oxidative stress decreases estradiol levels through increasing apoptosis of granulosa cells (Tripathi et al., 2013). Also, oxidative stress results into poor oocyte quality due to increase in caspase-mediated apoptosis of mature oocyte (Prasad et al., 2016) . Furthermore, BDNF is also locally expressed in the ovary and it is mainly involed in the folicular growth and oocyte maturation (Kawamura et al., 2005). A recent report has also demonstrated low levels of BDNF in oocytes isolated from PCOS women (Kowalska et al., 2015). In the same line, present study has also shown reduced BDNF and TrkB in PCOS rat ovary which could result into follicular impairment. Decresed estrogen and BDNF while increased oxidative stress and inflammation observed in the present study could result into defective follicular development and increased granulosa cell-death reasulting into ovarian cyst formation. Furthermore, newer reports also indicate that oxidative stress and inflammation may lead to epigenetic modification (Eini et al., 2017) and increased expression of microRNAs (miR21 & miR146a) (Salimi-Asl et al., 2016) which result into DNA damage and poor oocyte quality in PCOS.

The brain consumes high amount of oxygen for rapid production of ATP, which makes it vulnerable to oxidative stress and damage. Also, neurons have very poor antioxidant defence systems and thereby, largely depend on astrocytes and oligodendrocytes. Recent study has demonstrated that increased oxidative stress and inflammation in hypothalamus and other CNS areas may result into impaired carbohydrate and lipid metabolism and imbalance of energy homeostasis, leading to higher susceptibility for development of metabolic syndrome (Cai & Liu, 2012). Present study has clearly demonstrated significant increase in oxidative stress and inflammation in various regions of brain including pituitary, hypothalamus, hippocampus and frontal cortex, suggesting that an imbalance of redox molecules of CNS could result into metabolic aberrations in PCOS. In this context, a recent study in young women with PCOS has demonstrated association between glucose hypometabolism and mild insulin resistance in certain areas of brain (Castellano et al., 2015), which may further increase AGE production, leading to oxidative stress.

Apart from this, several reports indicate the role of increased oxidative stress and inflammation into psychiatry and neurodegenerative disorders. Increased levels of NFκB, TNFα, IL1β and IL6 have been demonstrated from post-mortem studies on different regions of brain of patients with bipolar depression (Ortiz-Dominguez et al., 2007; Rao et al., 2010). Also, many studies indicate the role of inflammation in pathogenesis of depression (Maes,

2008; Miller et al., 2009; Black et al., 2015; Oakes et al., 2016). The pathway of inflammation and oxidative stress-mediated depression involves an interplay of a variety of molecules including neurotransmitters and neurotropins. It has been proposed that activated microglia due to peripheral or CNS inflammatory response generates inflammatory cascade leading to release of TNFα, IFNγ, IL1β, IL2 and IL6 as well as reactive oxygen and nitrogen species (ROS & RNS). These cytokines are known to induce activity of enzyme indolamine-2,3-dioxygenase (IDO) which degrades tryptophan into quinolinic acid. As tryptophan is the precursor of serotonin, increased IDO activity leads to decreased serotonin production (Schwarcz & Pellicciari, 2002; Dantzer et al., 2008). At the same time, quinolinic acid, a product of IDO increases glutamate release and its receptor expression. Furthermore, IL1 and TNFα also decrease serotonin levels by increasing the expression of serotonin reuptake transporter (SERT) through activation of p38 MAPK pathway (Zhu et al., 2006). The increased inflammatory cytokines as well as *IDO1* and *SERT* expression observed in the present study can be well correlated with the reduced levels of serotonin and increased glutamate and *NMDA* receptor in letrozole-induced PCOS model (Chapter 6). Furthermore, IL1 and TNFα decrease the expression of brain-derived neurotrophic factor (BDNF) and its receptor, tyrosine kinase-B (TrkB) (Wu et al., 2007). BDNF/TrkB is the major neurotropic factor which aids in neuronal growth, development as well as in neurogenesis and loss of these factors can lead to cell death and cognitive impairment (Wu et al., 2007; Dantzer et al., 2008; Koo & Duman, 2008; Miller et al., 2009). Also, increase in glutamate due to decreased glutamate reuptake and increased NMDA receptor activation leads to excitotoxicity. Glutamate excitotoxicity increases cell apoptosis and demyelination by decreasing BDNF/TrkB expression. The increased glutamate level as observed in PCOS animals may therefore decrease *BDNF*/*TrkB* expression which could result into neuronal cell death as evident by increased expression of cleaved caspase-3. In addition to glutamate, oxidative stress also independently decreases the expression of BDNF in brain regions (Kapczinski et al., 2008). Furthermore, studies have demonstrated that treatment with Monoamine oxidase inhibitors (MAOI) increase BDNF expression (Nagatsu & Sawada, 2006; Balu et al., 2008). Increased MAO activity was observed in PCOS condition (Chapter 5), suggesting increased production of free radicals, which may result into high oxidative stress, leading to decreased *BDNF* expression. MAO-A has been also linked with altered glutamate dysregulation that could alter synaptic plasticity causing cognitive impairment, neurodegeneration and major depressive disorders (Meyer, 2017). The above results indicate that increased oxidative stress

and inflammation decrease serotonin content and increased glutamate level, leading to decrease in *BDNF/TrkB* expression, further resulting into neuronal cell death and behavioural impairment. However, for a detailed understanding of this impairment, further studies are needed.

As PCOS is a multifaceted disorder, several studies indicate the involvement of psychological co-morbidities in PCOS women. The clinical features and health implications of PCOS may predispose to an impaired quality of life (QoL), leading to a loss of self-esteem, poor body image, and psychological morbidity (Ching et al., 2007; Sharma, 2015). It was also found that women with PCOS had a significantly poorer quality of life (QoL) when compared with age-matched population. This decreased QoL observed in PCOS, combined with poor coping strategies, can result in co-morbid psychiatric conditions such as major depressive disorders and anxiety (Azziz et al., 2016). Studies have reported that women with PCOS have an increased prevalence of mood disorders, including depression (26–40%), anxiety (11.6%) and binge-eating (23.3%) (Pasch et al., 2008; Kerchner et al., 2009; Goodarzi et al., 2011). In this regard, role of neurotransmitters has been indicated in mood and depression. Women with depression demonstrate reduced levels of serotonin, dopamine and norepinephrine. Also, animal models wherein these neurotransmitters were depleted exhibit impairment in behavioural tasks such as forced-swim test and tail-suspension test (Dunlop & Nemeroff, 2007; Maletic et al., 2007). The reduced levels of neurotransmitters observed in the present study (Chapter 6) can be well correlated with the neurotransmitters metabolites seen in depressed patients.

It should be noted that hyperandrogenic and hypoestrogenic condition persists in the PCOS condition (Chapter 3). It has been well known that estrogen acts as a major neuroprotectant (Solum & Handa, 2002; Brann et al., 2007) and alteration in its levels can lead to mood alteration in permenopausal period (de Novaes Soares et al., 2001). Direct incubation of estradiol with microglial cells inhibits phagocytosis and decreases production of ROS (Bruce-Keller et al., 2000). Also, estradiol is able to inhibit proinflammatory molecules including inducible nitric oxide synthase (iNOS) and prostaglandin E_2 (PGE_2), indicating that estradiol can act as an antioxidant as well as an anti-inflammatory agent (Kato et al., 2013). These neuroprotective effects of estradiol are mediated through estrogen receptors (ERα & ERβ) (Sarvari et al., 2011). Furthermore, Estradiol directly stimulates the expression of BDNF mRNA via binding to ERα (Solum & Handa, 2002). The decreased mRNA of *BDNF*

observed in the present study may be partly due to reduced estrogen and ERα expression in PCOS (Chapter 3). Also, a recent study has demonstrated that estradiol treatment decreases hippocampal inflammation and it also reduces depression-like behaviour in mice (Xu et al., 2016). The reduced levels of estradiol in PCOS may result into decreased neuronal protection which could culminate into depressed mood. However, the exact role of estrogen and its responsiveness in context of oxidative stress and mood disorders in PCOS warrants further investigations.

The present study clearly demonstrates participation of oxidative stress and inflammation in letrozole-induced PCOS rat model. The increased oxidative stress and inflammation with reduced expression of BDNF in ovary may result into follicular impairment and increased follicular atresia in PCOS condition. Along with the ovary and systemic level elevation of oxidative stress, current data for the first time demonstrates increased oxidative stress in brain regions of PCOS condition. The increased oxidative stress was evident by decreased activity of SOD, Catalase and GPx with low levels of glutathione which resulted in increased lipid peroxidation and protein carbonylation. Also, transcripts of proinflammatory cytokines Tnf, Ifng, Il1b, Il2, and Il6 were markedly elevated whereas anti-inflammatory Il10 was reduced in brain regions of PCOS animals. Furthermore, the increased oxidative stress and inflammation increases IDO and SERT expression while reducing BDNF/TrkB expression, which results into decreased monamine levels and increased glutamate excitotoxicity further resulting into neuronal cell loss. In addition, the alterations observed in the PCOS brain mimic the microenvironment of patients suffering from major depressive disorders. In this line, data from the current study aptly demonstrates behaviour impairment in letrozole-induced PCOS rats, thereby indicating that change in the redox environment of the brain may alter several signalling processes leading to depression.

Current study depicts PCOS as being associated a pro-inflamed state wherein the key gonadal tissues as well as neuronal tissues are greatly affected. Also, this study for the first time, has implicated that depression in PCOS is a condition arising out of modulations in several factors including hormones, neurotransmitters and neuropeptides. Moreover, results so far demonstrate a disturbance in several important aspects that could contribute to the etiology of PCOS. These clues can be further explored to get an insight into the molecular aberrations result into PCOS pathology.