PhD Research Proposal

Evaluation of the ameliorative effects of the active ingredient from the fruit pulp of *Eugenia jambolana* against adverse effects in rat Leydig cells induced by Oxidative stress

Testis is the primary sex organ in males and has two important functions – spermatogenesis and steroidogenesis, occurring in two different compartments of the testis – seminiferous tubules and the interstitium respectively. Impairment of testicular function can be attributed to changes in either the seminiferous tubules (which harbour the tubular germ cells at various stages of spermatogenesis) or the interstitial tissue (which harbours the Leydig cells responsible for secretion of testosterone (steroidogenesis)).

**Review of Literature - Chronology**

| 1980 – 1990 | • NAC evolved as a compound with immense therapeutic value in cases of acetaminophen overdose and pulmonary disorders by functioning as a redox buffer as well as an ROS scavenger.  
• H$_2$O$_2$ found to have an antigonadotropic activity in rat ovarian cells. |
| 1990 – 2000 | • H$_2$O$_2$ found to have an antigonadotropic and antisteroidogenic activity in rat granulosa cells by inhibiting the activity of one or more of steroidogenic enzymes involved in cholesterol metabolism. |
- NAC found to be an effective inhibitor of cell death in several systems including cell lines and semen, as a treatment against HIV and also effective in scavenging reactive oxygen species.

- Reactive oxygen species (ROS) found to be involved in increasing testicular apoptosis following acute experimental torsion and heat stress.

<table>
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<th>2000 – 2010</th>
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<td>• H$_2$O$_2$ found to induce oxidative stress and apoptosis at physiological concentrations in Leydig cells and testicular germ cells via various apoptotic pathways.</td>
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<td>• ROS found to be involved in Leydig cell apoptosis via extrinsic pathway and JNK pathway following repeated hCG administration.</td>
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<td>• <em>Eugenia jambolana</em>, a natural flavonoid containing plant found to have an anti hyperglycaemic and hypolipidemic property in experimental animals and human adults.</td>
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The process of testosterone production (steroidogenesis) is oxygen dependent and all testicular cell membranes being rich in polyunsaturated fatty acids (PUFA) are highly prone to Reactive Oxygen Species (ROS) attack (Peltola et al., 1992). Reactive Oxygen Species (ROS) are produced continuously in cells as the by-products of mitochondrial and microsomal electron transport reactions and other metabolic processes. In addition, analysis of the structural features of the testes reveals a close association between the testicular macrophages and
Leydig cells in the interstitial tissue. It is known that, upon activation, macrophages release cytokines and ROS to counteract the stimulus. Testicular macrophages are known as the store and production house for free radicals. ROS cause tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation, depletion of cellular thiols, and activation of pro-inflammatory cytokine release.

As compared to several other ROS, $\text{H}_2\text{O}_2$ has a lower biological activity and possesses the ability to cross membranes and diffuse away from the site of generation. These attributes, suggest the possible role of $\text{H}_2\text{O}_2$ as a potent signal transduction molecule. There is a growing literature showing that $\text{H}_2\text{O}_2$ can be used as an inter- and intra-cellular signaling molecule. At sites of inflammation, $\text{H}_2\text{O}_2$ generated by activated phagocytes appear to modulate the inflammatory process, e.g. by up-regulating expression of adhesion molecules, controlling cell proliferation or apoptosis and modulating platelet aggregation. $\text{H}_2\text{O}_2$ is a very potent form of reactive oxygen species, the implications of which in affecting normal testicular functions have been well documented by several earlier studies as described under. The antisteroidogenic actions of hydrogen peroxide on rat Leydig cells have been studied. Gautam et al., (2006) have reported that $\text{H}_2\text{O}_2$ affects the normal activity of $3\beta$- hydroxysteroid dehydrogenase suggesting the involvement of $\text{H}_2\text{O}_2$ in affecting normal steroidogenesis in rat Leydig cells. It has been shown that hydrogen peroxide acts directly on rat Leydig cells to diminish testosterone production by inhibiting P450scc activity and StAR (steroidogenic acute regulatory protein) protein expression (Tsai et al, 2003). Hydrogen peroxide has also been shown to have marked antigonadotropic activity in human luteal and granulosa cells and blocks basal and stimulated progesterone production (Behrman
and Preston, 1989; Margolin et al, 1990) by inhibiting the activity of one or more of the steroidogenic enzymes involved in cholesterol metabolism (Endo et al 1993). H₂O₂ has been shown to cause apoptosis and oxidative stress in Leydig cells in rats leading to modulation of Leydig cell functions at physiological concentrations (Gautam et al., 2006), however, the pathways associated with H₂O₂ mediated Leydig cell apoptosis are largely unknown. Involvement of ROS has also been suggested in Leydig cell apoptosis due to a non oxidant stimulus in the form of repeated hCG administration leading to apoptosis mainly via the extrinsic pathway and JNK (Aggarwal et al., 2009).

Apart from the interstitial Leydig cells, the tubular germ cells have also been reported to be affected by hydrogen peroxide. Ikeda et al (1999) have shown that rat testicular germ cells undergo apoptosis following exogenous supply of oxygen free radical generating xanthine and xanthine oxidase which lead to the generation of hydrogen peroxide. Maheshwari et al. (2009) have demonstrated the induction of various apoptotic pathways involved in testicular germ cells following intervention with hydrogen peroxide (1-10 µM) in vitro.

Therefore, ROS are important for normal testicular functions, however, an excess of ROS can lead to structural and functional dysfunction of the testes and increase in testicular cell apoptosis. Protection of sperm from ROS damage by antioxidants (including NAC) as well as the preventive effects of oxygen radical scavengers on testicular function after acute experimental torsion (Oeda et al, 1997; Prillaman et al, 1997) suggests that antioxidants act as factors of survival, ensuring germ cell function. Thus, it would be worthwhile to investigate the effect of new and potent antioxidant on testicular cell survival during and after the induction of hypospermatogenesis. Thus it would be interesting to find the
ameliorative effect of antioxidant during H$_2$O$_2$ treatment to testicular Leydig cells \textit{in vitro}.

\textit{N}-Acetyl-\textit{l}-cysteine (NAC) is a well established thiol antioxidant that after uptake, deacetylated, and converted to glutathione, functions as both a redox buffer and a reactive oxygen intermediate scavenger (Roederer et al, 1992; Mayer et al, 1994; Burgunder et al, 1989). In experimental studies, NAC has been reported to be an effective inhibitor of physiological cell death in several systems (Mayer et al, 1994; Cossarizza et al, 1995; Delneste et al, 1996). In addition, it is a compound with which there is extensive clinical experience in the treatment of patients suffering from acetaminophen overdose, pulmonary disorders, or acquired immunodeficiency syndrome (Roederer et al, 1992; Burgunder et al, 1989; Moldeus et al, 1986) Earlier unpublished data suggests the efficacy of NAC in minimizing cell apoptosis mediated by exposure to hydrogen peroxide in germ cells and also in Leydig cells after repeated stimulation with hCG.

Besides synthetically derived antioxidants like NAC, natural compounds like flavonoids, the phenolic substances isolated from plants have also been known to possess antioxidant properties, due to their ability to reduce free radical formation and to scavenge free radicals, thus, increasing the interest in them as preventive and therapeutic agents. \textit{Eugenia jambolana} (Myrtaceae, common name: Black plum/Black berry in English and Jamun/Jambul in Hindi) is one such plant, seeds of which have been found to have a hypoglycemic, anti-inflammatory, neuropsychopharmacological, anti-bacterial, anti-HIV, anti-diarrhoeal effect (Chaturvedi et al., 2007) and an antioxidant effect (Vasi et al., 2009). Sharma et al., isolated the active ingredient from the fruit pulp of \textit{Eugenia jambolana} (\textbf{Indian Product Patent 2,30,753; February 2009}) and have found it to have an
antihyperglycemic effect (Sharma et al., 2006). Sharma et al (2010) have reported the antihyperglycemic and hypolipidemic activity of the aqueous and ethanolic extracts of seeds administered orally to experimental animals and to human adults at various dose levels. However, no preliminary information is available on the anti-apoptotic and anti-oxidative property of the active ingredient from the fruit pulp extract. Thus it is of interest to investigate the same against oxidative stress and apoptosis in rat Leydig cells and compare the effect with that of an established antioxidant, NAC.
General objective of the study:

To evaluate the ameliorative effects of the active ingredient from the fruit pulp of *Eugenia jambolana* (EJE) in rat Leydig cells against oxidative stress.

Specific objectives of the study:

1. To study dose and time kinetics of the ameliorative effect of the active ingredient from the fruit pulp of *Eugenia jambolana* on the adverse effect of \( \text{H}_2\text{O}_2 \) on isolated rat Leydig cells and study the molecular mechanisms associated with the cell survival.

2. To evaluate the beneficial effect of the extract against oxidative stress induced *in vitro* or *in vivo* and compare the same with that of the established antioxidant effect of NAC.

Proposed Work Plan:

1. Testicular cell isolation/viability/purity:
   a. Isolation of leydig cells.
   b. Leydig cell viability test by Trypan blue dye exclusion test.
   c. Leydig cell purity determination by 3β-HSD Staining.

2. Dose and Time Kinetics of treatment:
   a. Standardization of the effective dose of the extract.
   b. Treatment with various doses of the extract and for various exposure times with co-incubation with \( \text{H}_2\text{O}_2 \) for Leydig cells.

3. Measurement of Oxidative stress parameters:
a. **Lipid peroxidation** (Ohkawa et al., 1979): Degree of lipid peroxidation can be assayed by estimating the amount of thiobarbituric acid reactive substances (TBARS) by Spectrophotometric method. It is based on the reaction of thiobarbituric acid (TBA) with malonaldehyde (MDA), one of the aldehyde products of lipid peroxidation. The amount of MDA-TBA bi-product produced following the reaction is measured at \( \lambda \) 532 nm.

4. **Measurement of antioxidant enzymes in treated Leydig cells:**

a. **Superoxide dismutase (SOD)** (Das et al. 2000): Are metallozymes that catalyze dismutation of superoxide anion into oxygen and hydrogen peroxide. Superoxide dismutase scavenges superoxide radicals that are produced by photo-reduction of riboflavin. These superoxide radicals are then allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with napthylamine to produce a red azo compound whose absorbance is measured at 543 nm.

b. **Catalase Activity** (Aebi, 1974): Present in the peroxisomes of nearly all aerobic cells and serves to protect the cells from the toxic effects of hydrogen peroxide by catalyzing decomposition of \( \text{H}_2\text{O}_2 \). Catalase exerts a dual function: 1) decomposition of \( \text{H}_2\text{O}_2 \) to give \( \text{H}_2\text{O} \) and \( \text{O}_2 \) (catalytic activity) and 2) oxidation of H donors, e.g., methanol, ethanol, formic acid, phenols with the consumption of 1 mol of peroxide (peroxidic activity). In the ultraviolet range \( \text{H}_2\text{O}_2 \) shows a continuous increase in absorption with decreasing wavelength. Thus,
any decomposition of H$_2$O$_2$ can be correctly followed by monitoring the decrease in absorbance (λA 240) of H$_2$O$_2$ per unit time. This change in absorbance is directly proportional to the measure of catalase activity.

c. **Glutathione-S-Transferase (GST)** (Habig et al., 1974): This enzyme belongs to super family of inducible enzymes, which catalyze the conjugation of reduced glutathione to various electrophilic substrates and are important in detoxification of many xenobiotics in mammals. The activity of glutathione-s-transferase will be measured by thioether formation between GSH and 1-chloro-2,4-dinitrobenzene(CDNB) at 340 nm.

d. **Determination of Glutathione and total sulfhydryl group content:** Glutathione (GSH) react directly with hydrogen peroxide and superoxide anion, hydroxyl and alkoxyl radicals by its free sulphydryl groups and plays an important role in the protection against damage produced by oxidants and free radicals GSH acts to preserve SH groups of protein in the reduced state by means of disulfide interchange. The maintenance of free protein sulfhydryl groups is important in the proper folding and activity of protein.

e. **Total antioxidant capacity:** Can be determined spectrophotometrically by calculating the ability of antioxidants to scavenge the 2,2'-azinobis (3-ethylbenzothiazoline 6-sulphonate) (ABST) radical cation, ABST$^+$, inhibiting its absorption at 734 nm.
5. Detection of Apoptosis in extract/H₂O₂ treated Leydig cells:

a. **DNA ladder assay:** Apoptosis and cell mediated cytotoxicity are characterized by a fragmentation of the genomic DNA. These DNA fragments have a length of about 180 base pairs or multiples thereof (360, 540, 720, ...), the characteristic DNA-length of a nucleosome (DNA-histone-complex). Endonucleases selectively cleave DNA at sites located between nucleosomal units (linker DNA). In agarose gel electrophoresis these DNA fragments are resolved to a distinctive ladder pattern.

b. **TdT – mediated deoxyuridinetriphosphate nick end labeling (TUNEL):** TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage. Cells fixed on poly-L-lysine coated slides would be used for the TUNEL assay.

c. **Comet Assay:** This helps to detect DNA single strand breaks. This provides a simple and effective method for evaluating DNA damage to cells using fluorescence microscopy.
6. Study of Molecular Mechanism associated with extract mediated attenuation of Leydig cell apoptosis:

a. *PCR and Western Blotting:* mRNA and protein expression for PARP, Caspase 9, Bid, Bak, Bad, JNK, NFκB, Cyt C, Fas, FasL, p53, Caspase 8, JNK, Bcl2 and internal control (β-actin/GAPDH).

**Acknowledgements**

A generous gift of the active ingredient from the fruit pulp of *Eugenia jambolana* (EJE; *(Indian Product Patent 2,30,753; February 2009)*) to Dr. M. M. Misro, Professor, Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Delhi, from Dr. Suman Bala Sharma, Professor, Department of Biochemistry, University College of Medical Sciences, University of Delhi, Delhi, is greatly acknowledged.
Work Plan

Isolation of Rat Leydig cells

- Test Viability – Trypan Blue Dye Exclusion
- Test Purity – 3β HSD staining

Induction of oxidative stress in Leydig cells in vitro by H₂O₂ exposure

Measurement of Oxidative Stress Parameters

- Degree of Lipid Peroxidation
- Levels of Antioxidant Enzymes
  - SOD
  - Catalase
  - GST
  - Total Antioxidant Capacity (TAC)
  - Total Cellular Glutathione (GSH)

Biochemical Assays

mRNA & Protein Expression

Oxidative stress leading to Apoptosis

Evaluation of extent of Apoptosis and ameliorative effect of EJE on the inhibition of oxidative stress induced apoptosis on exposure to H₂O₂ in vitro

- Degree of Apoptosis
  - TUNEL Assay
  - COMET Assay
  - DNA Ladder Assay

- Estimation of various Apoptotic Markers
  - Colorimetric Assays
  - mRNA & Protein expression of markers of different pathways of Apoptosis
  - Caspase-3
  - Caspase-8
  - Caspase-9

NB: Similar in vivo interventions will be planned to induce oxidative stress in Leydig cells and the ameliorative effect of the extract will be evaluated against the same.