SUMMARY OF CHAPTERS

Chapter: 1 Chromium (VI) induced oxidative stress in Testes and Epididymis: Realistic dosage and duration dependent study and, role of Melatonin

Adult wistar(around 180 days old) rats have been exposed through drinking water to Cr(VI) (20mg/ kg BW/ day) with or without simultaneous melatonin(10mg/ kg BW/ day) administration for three treatment schedule(15, 30 and 60days). Administration of melatonin was carried out in the evening at 18.00hrs and the animals maintained under normal light and dark regime(L:D 13:11). All animals were assessed for the levels of enzymatic(CAT, SOD, GPx and GR) and non-enzymatic(GSH and Ascorbic acid) and degree of lipid peroxidation(LPO) and tissue metal load in testis and epididymis and serum melatonin titre. Results of the current study demonstrate the potential of hexavalant chromium to generate excessive ROS leading to impaired oxidative status of male reproductive organs. Decreased levels and availability of non-enzymatic(ascorbic acid and GSH) and enzymatic (SOD, CAT, GPx and GR) antioxidants respectively in testis and epididymis of Cr treated rats could be held responsible for the increased LPO levels recorded in the present study by way of increased ROS formation. The recoded decrease in the level of GSH of both testis and epididymis of rats, subjected to Cr(VI) exposure in the study could adversely affect the activities of GSH dependent enzymes. An interesting observation of the present study is that while the non-enzymatic antioxidants(GSH and ascorbic acid) showed a duration(chromium exposure) dependent linear decrease, there was no such linear effect on the enzymatic antioxidants (CAT, SOD, GPx and GR). Along with the

endogenous tissue GSH and ascorbic acid, melatonin also seems to play a contributing role in lessening the impact of the indole in a progressive duration dependant manner. It is also of interest to note that, of all the antioxidant enzymes, except for activity levels in epididymis compared to testis. This may be relevant in terms of epididymal functions related to maturation, decapacitation and integrity of sperms. Another significant observation is the resistance of testicular GPx activity to Cr toxicity and may be looked upon as an adaptive mechanism to protect testis against peroxide radicals which could probably interfere with the vital processes of spermatogenesis and steroidogenesis. The findings of the present study suggest that, co-administration of melatonin along with chromium can exert marked protection against chromium induced oxidative insult. Importantly, co-administration of melatonin shows well maintained pool of enzymatic (SOD, CAT, GPx and GR) and non-enzymatic(GSH and ascorbic acid) antioxidants, reflecting the relatively healthy antioxidant milieu of testis and epididymis compared to animals treated with Cr(VI) alone. Current findings suggest increased oxidative stress due to ROS generation to be responsible for increased lipid peroxidation and impaired antioxidant cellular defense in epididymis and testis of hexavalent chromium treated animals in a duration dependent manner. Results of the present study advocate the use of melatonin as a potential antioxidant therapeutic.

Chapter: 2 Cadmium induced oxidative stress in Testes and Epididymis: Realistic dosage and duration dependent study and, role of Melatonin

Cadmium a potential environmental toxicant is present in high amount in the dietary sources of the Vadodara population. In an attempt to assess the Cd induced male reproductive toxicity, wistar rats of around 180 days of age were exposed to Cd(9 mg/

kg BW/ day) for 15, 30 and 60 days. Concurrently, the efficacy of the melatonin(10 mg/ kg BW/ day) as a protectant against Cd induced insult of testis and epididymis was also studied. After the treatment animals were sacrificed and the testis and epididymis were used for biochemical estimations of lipid peroxidation and enzymatic(CAT, SOD, GPx and GR) and non-enzymatic(GSH and Ascorbic acid). This study clearly indicates duration dependent increase in oxidative stress in both testis and epididymis of Cd exposed rats. Significantly, duration dependent increase in LPO to the tune of more than 50% in the case of testis and more than 80% in the case of epididymis provides validity. A comparison of duration dependent effects of Cd in testis and epididymis clearly reveals much higher lipid peroxidation in epididymis. Though there is minimal LPO in testis during short duration exposure to Cd, increasing oxidative stress is nevertheless indicated by the significantly inhibited activities of SOD and CAT and marked depletion of ascorbate. The increasing LPO on along duration exposure to Cd seems to be due to the significant inhibitory effects on GPx and GR and highly depleted GSH content. Noticeably higher lipid peroxidation seen in the epididymis on short duration exposure of Cd along with marked depletion in GSH content might suggest depletion of –SH groups and release of copper and iron from membrane proteins and enzymes and the resultant ROS formation as suggested above as the cause of oxidative stress. Clearly, co-administration of melatonin along with Cd could effectively prevent and/ or minimize the alteration in endogenous antioxidants caused by cadmium. This is further supported by our observation of significantly reduced serum melatonin titre in cadmium intoxicated animals. Overall, the present study brings out (I) a generalize Cd induced reproductive toxicity by oxidative stress, (II) differential mechanism of Cd toxicity in testis and epididymis on short term verses long term exposure and (III) the

significant role of melatonin as a protectant and a possible future therapeutic agent worthy of testing against metal toxicity.

Chapter 3: Nickel induced oxidative stress in Testes and Epididymis: Realistic dosage and duration dependent effect and, role of Melatonin

To assess the toxicity of nickel on male reproductive system, around 180 day old rats of wistar strain were exposed to Ni(40mg/ kg BW/ day) through drinking water for three different treatment schedule(15, 30 and 60 days). In order to check the protective action of melatonin animals were simultaneously treated with intraperitoneal injection of melatonin(10 mg/ kg BW/ day). The completion of treatment animals were sacrificed and the testis and epididymis were used for biochemical estimation of lipid peroxidation and antioxidants(both enzymatic – CAT, SOD, GPx and GR and non-enzymatic – GSH and Ascorbic acid) together with tissue metal load and serum melatonin titre analysis. Present has revealed pronounced Ni induced oxidative stress in both testis and epididymis marked by duration dependent increase in LPO and depletion and inhibition of endogenous non-enzymatic and enzymatic antioxidants respectively. The markedly significant inhibition of SOD activity in both testis and epididymis compared to all other enzymes indicates its greater vulnerability under Ni intoxication. Nickel induced decrement in antioxidant machinery of reproductive organs and thereby contributes to a heightened oxidative stress, which is reflected in a duration dependent increase in both testis and epididymis. On a comparative note based on the observations of this study, epididymis seems to be more dependent on GSH and GPx rather than ascorbic acid as in testis. Ni induced inhibition of antioxidant machinery seems to be of greater severity in epididymis which is well reflected in the recorded higher levels of LPO

and, this might bespeak of nickel mediated interference in epididymal functions related to sperm maturation and motility. The results obtained suggest that melatonin has a highly potent ability not only to upregulate the antioxidant compounds when administered alone but also to protect and/ or nullify Ni induced inactivation/ inhibition of antioxidant machinery when co-administered with the metal. It si also clear that melatonin is efficient in nullifying duration dependent effect of Ni. Overall, the present study records Ni induced oxidative stress in testis and epididymis with slightly differential adaptive mechanisms and relatively greater sensitivity of epididymis. The role of melatonin as an effective protctant against Ni induced oxidative stress is also brought out.

Chapter 4: Trimetallic induced oxidative stress in Testes and Epididymis: Realistic dosage and duration dependent studies and, role of Melatonin

Male reproductive toxicity of a trimetallic mixture(TM) of Cr(20mg/ kg BW/day), Cd(9mg/ kg BW/ day) and Ni(40mg/ kg BW/ day) and putative protective role of melatonin(10mg/ kg BW/ day) have been assessed on around 180 days old wistar rats for 15, 30 and 60 days. Trimetallic mixture and melatonin(at 18.00hrs) were administered through drinking water and intraperitoneally respectively. Exposure to the TM mixture has recorded significant oxidative stress marked by increasing LPO and decrease in both non-enzymatic and enzymatic antioxidants. Compared to testis, epididymis has shown significantly greater degree of LPO and decrement in antioxidants. The maximal induction of LPO on exposure to TM mixture is a reflection of the cumulative effect of all the three metals, especially Cd and Cr in generating ROS like superoxide anion, hydrogen peroxide and hydroxyl radicals. The

greater inhibition of SOD seen with TM mixture can not only be related with the persistent dismutation of superoxide anion but also by the inactivating effect of Cd by displacement of Zn from Cu-Zn-SOD(cytosolic or SOD1). The indoleamine, melatonin used as a supplement in the present study to evaluate its potential to protect testis and epididymis against the TM mixture induced pro-oxidant state. Melatonin has been found to be very effective in controlling LPO, in preventing depletion of GSH and in resisting inhibition/ inactivation of SOD and GR. Even in the case of ascorbate depletion and decreasing in activities of CAT and GPx, melatonin seems to exert reasonably good protective effect. The comparison has also revealed that though it has an overall redeeming effect against the TM mixture in both testis and epididymis, its competence against individual metals seems to be of the order Cr< Cd< Ni in the case of testis and Cd< Ni< Cr in the case of epididymis. In conclusion, it is inferable from the present study on exposure to a TM mixture that, the resposes are not only tissue specific but also a consequence of complex interactions between the constituent metals. It is also inferable from the present study that, melatonin could be considerable as a potent therapeutic agent against metal intoxication either used alone or in combination with vitamins.

Chapter 5: Chromium (VI) toxicity and protective effect of melatonin: *In vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In vitro* alterations in cell viability and testosterone secretion

Chromium(VI) is a major metal pollutant whose systemic toxicity has been studied to a greater extent. Reproductive toxicity of the male system has however received much lesser attention compared to other organs. In this context, the present evaluation assesses the impact of such realistic dosage of Cr(VI) on structural and functional

alteration in testis and epididymis. In continuation, the male adult wistar rats were treated with Cr(VI) (20mg/ kg BW/ day) for three different duration(15, 30 and 60 days) and also putative protective role of evening melatonin(10mg/ kg BW/ day) was tested. The observed changes in testis involve a progressive disruption in spermatogenesis marked by initial sloughing off of spermatozoa, followed by gradual loss of spermatids and post-pachytene spermatocytes. The present study on chronic Cr exposure has revealed marked structural disorganization of the cauda epididymis. Not only are there complete disappearance of spermatozoa from the lumen of epididymal tubules (with only presence of damaged apoptotic or necrotic germ cells) but also tubular epithelial disruption and appearance of vacuole like structures within the epithelium. Apart from the observed structural alterations in epididymis and in the testis causing disruption in spermatogenesis, the steroidogenic potential of the testis was also compromised. From the observations and recorded data, it becomes clear that Cr(VI) exposure leads to progressive decrease in 3 β HSD and 17 β HSD activities and fall in serum testosterone and estrogen titres. The Cr(VI) induced toxicity effects on testis and epididymis are well reflected in the quantitative changes affecting sperm count decreased by 42% , sperm motility test showed 35% increase in immotile sperms with about 12% in the occurrences of abnormal sperms. In the present evaluation melatonin has been found to afford protection against almost all the parameters of in vivo experiment. To confirm and strengthen the in vivo observations, in vitro studies on cell viability, basal and hCG stimulated testosterone secretion have been assessed in isolated Leydig cells. The results from these assays clearly reveal increasing cytolethality in temporally increasing course of Cr exposure with about 48% lethality with in 12 hrs. Interestingly, in Cr exposed experimental Leydig cells, the increase in testosterone secretion upon hCG stimulation showed a decrement from

55% at 3 hrs to 26% at 6 hrs. Again, Cr exposed cell in presence of melatonin showed a 4% increase from 3-6 hours under hCG stimulation compared to 11% without melatonin. This 4% increase in Cr + Mel cells is very much identical to the increase shown by control Leydig cells under hCG stimulation from 3-6 hours. This is probably the first report brining out the new dynamics of testosterone secretion influenced by melatonin in Cr stressed Leydig cells. A novel finding that comes out of this study is that the dynamics of testosterone secretion by melatonin under Cr toxicity or stress is quite different from the non-stressed condition.

Chapter 6: Cadmium toxicity and protective effect of melatonin: *In Vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In Vitro* alterations in cell viability and testosterone secretion

In the present investigation, an attempt is therefore made to evaluate the duration dependent (15, 30 and 60 days of treatment) effect of Cd(9mg/ kg BW/ day) through drinking water on steroidogenic enzyme activity, serum testosterone and estradiol titre, semen parameters (sperm count, sperm motility and sperm abnormality) along with the effects on cell viability and testosterone production under basal as well as stimulated conditions by Leydig cell primary culture. Concurrently, the putative protective role of melatonin(10mg/ kg BW/ day, *i.p.* injection) on the above mentioned parameters, both *In Vivo* and *In Vitro,* has also been assessed. The present study reveals deleterious effects of Cd on spermatogenesis and steroidogenesis in the testis and, on functional competence of the epididymis. The recorded decreased activity of 3β and 17 β HSD, clearly reflected in lowered serum testosterone and estrogen titres. In accordance with these, is the observation of the significantly decreased epididymal sperm count? Moreover, the increasing percentage of sperm

abnormalities despite improving motility may indicate extreme vulnerability of mature sperms within the testis and epididymis to the direct or indirect modes of Cd induced oxidative stress. Though melatonin has a slight lowering effect on serum testosterone and testicular 3β and 17β HSDH activities in control animals, there was nevertheless favorable influence on sperm motility and sperm abnormality. But when given along with Cd, it has shown substantial protective effect on enzyme activity, steroid hormone levels, sperm count, motility and abnormality. The effect of Cd is still discernable suggesting that even at the dose of 10mg/kg, melatonin is not fully effective in checking the manifestations of metal toxicity. It is inferable that, while melatonin is effective in minimizing oxidative stress, it is incapable by itself to resist totally the influence on biochemical and functional features of testis and epididymis. The significant decrement in serum titre of melatonin seen between 15 to 60 days of Cd exposure implies its involvement in resisting Cd induced oxidative stress and differential tissue effects. The observed *In Vivo* effects of Cd on Leydig cell steroidogenesis was further assayed in an *In Vitro* system of cultured Leydig cells along with cell viability. The assay shows that there is increasing cytolethality with increasing duration of Cd exposure (20%-30% from 3-12 hours). Concurrent presence of melatonin afforded significant protection against Cd induced cytoletahlity and the percentage of viable cells is significantly higher. Release of testosterone from cultured Leydig cells is significantly affected by the presence of Cd as under basal conditions testosterone secretion decreased by 40% at 3 hrs to about 48% at 6 hours. Even under hCG stimulation, Cd decreased testosterone secretion by 50% at 3 hours to 55% at 6 hours. Obviously, an immediate consequence to Cd toxicity is significantly reduced testosterone secretion probably attributable to the metal induced oxidative stress. In conclusion, the present *In Vivo* and *In Vitro* studies suggest differential acute versus

chronic response to Cd with heightened testicular and epididymal damage on a short term basis and/an adaptive mechanism resisting and reverting the damages in a qualitative sense on a long term basis. Melatonin is able to protect to a great extent the alterations induced by Cd and a therapeutic combination of melatonin supplemented with other vitamins may prove to be the mode of combating Cd induced reproductive toxicity. Further, it is also revealed that melatonin dynamics on steroidogenesis in the Leydig cells are different and it seems that melatonin has a positive favorable influence under metal stressed state.

Chapter 7: Nickel toxicity and protective effect of melatonin: *In Vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In Vitro* alterations in cell viability and testosterone secretion

Toxicity of nickel(40mg/ kg BW/ day) on male reproductive system of a physiologically relevant animal model(*wistar* rat) for three different duration(15, 30 and 60 days) has been assessed in terms of serum titres of steroids, activity of seroidogenic enzymes and qualitative and quantitative effects on sperm by studies were also extended to assess the effect of Ni on *In Vitro* testosterone production by isolated rat Leydig cells under basal and stimulated conditions. Male reproductive toxicity of Ni is evident from the herein recorded changes in steroid dehydrogenases, serum T and E_2 levels, sperm count, sperm motility, sperm abnormality and histologically noticeable lesions in testis and epididymis. Disruption in spermatogenesis is marked by loss of sperms and spermatids in most of the tubules. Apart from its effect on seminiferous tubules, Ni also seems to affect the Leydig cells and the process of steroidogenesis as, the activities of 3-β and 17-β HSDs and serum T and E_2 levels are all significantly compromised. A fall in serum and intratesticular T

levels together can also contribute to arrest in progession of spermatogenesis as some of the stages are vitally androgen dependent. Higher degree of inhibition of the steroidogenic enzymes occurs during the longer durations of exposure (30 and 60 days) indicating the increasing toxicity of Ni on the biochemical make-up of Leydig cells. Exogenous melatonin administration tried out as a protectant antidote against Ni toxicity has revealed its favorable effect on almost all the parameters affected by Ni. The observed *In Vivo* effects on steroidogenesis have been tested on an *In Vitro* systems of cultured Leydig cells. Cell viability assessed at the end of 3, 6 and 12 hours of Ni exposure of shows a gradually increasing cytotoxicity of 13% at 3 hours, 19% at 6 hours and 24% at 12 hours. In conclusion, the present study has shown Ni induced oxidative stress and deleterious effects on steroidogenesis and spermatogenesis as well as on epididymal functions and quantitative and qualitative aspects of sperm. Melatonin has been found to be effective in preventing these effects though not completely.

Chapter 8: Trimetallic mixture toxicity and protective effect of melatonin: *In Vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In Vitro* alterations in cell viability and testosterone secretion

The present study intends to evaluate, toxicity manifestations on exposure to the metallic mixture(Cr(VI)-20mg/ kg BW/ day, Cd-9mg/ kg BW/ day and Ni-40mg/ kg BW/ day) in terms of spermatogenesis, steroidogenesis, sperm maturation, activity of steroids dehydrogenases enzymes (3β HSD and 17β HSD), serum titres of testosterone and estradiol and sperm parameters (epididymal sperm count, motility and abnormality), in a temporal sequence of exposure to the metallic mixture. Further,

to confirm the results of *In Vivo* findings, a primary culture of isolated Leydig cells was challenged to the metal mixture under *In Vitro* conditions in terms of cell viability and, testosterone production under basal and hCG stimulated conditions. Interestingly, this would be the first investigation of its kind in which the effects of natural route of exposure (oral route) of a realistic dosage of metal combination on serum NO levels and apoptosis in testis (TUNEL assay and Caspase3 activity) are being explored. Moreover, antioxidant property of melatonin, was assessed to check its protective ability against the multiple metal induced functional derailment of male reproductive organs and altering serum hormonal and clinical chemistry parameters. The histopathological observation on testis and epididymis suggest marked inhibitory effects on testicular spermatogenesis and epididymal functions in relation to sperm morphology and maturation. Most of the seminiferous tubules show disturbed spermatogenesis with loss of sperms, spermatids and spermatocytes. The epididymal epithelium appears hypotrophied. These deleterious effects of trimetallic mixture on testis and epididymis are clearly reflected in the recorded significant decrease in epididymal are clearly reflected in the recorded significant decrease in epididymal sperm count and motility as well as on the increasing percentage of abnormal sperm of morphologically abnormal sperms. The interactive effects of metals on epididymal functions can also be ganged from the comparative slope line graph of an antagonistic effect of Ni over Cr and Cd at short and medium durations of exposure and a synergistic effect of all the 3 metals on longer duration and an increasing additive effect from short to long duration exposure on the percentage increase of abnormal sperms. The detrimental T/E_2 ratio, contributed by the pronounced decrease in T secretion finds substantiation by the concurrently observed significant decrement in 3 β and 17 β HSD. The graphs on temporal slope for both the dehydrogenases and

testosterone clearly indicate an additive inhibitory effect of all the three metals especially at the longer durations of exposure. Apparently, the inhibitory effect of Cd, Cr and Ni on steroid dehydrogenases and testosterone is additive when present together and has a much higher inhibitory potential compared to that of the metals individually. The *In Vitro* evaluation using isolated Leydig cells reveals greater degree of cytolethality compared to the degree seen with Cr, Cd and Ni individually. Melatonin added simultaneous to the trimetallic mixtures exerts protective effect on the metal mixture induced cytolethality as can be inferred from the observed increased cell viability. It can be concluded that in general, the trimetallic mixture of Cr, Cd and Ni has both synergistic and additive effects on most of the parameters assessed herein as well as antagonistic effect of Ni on epididymal functions. It is also evident that melatonin has a remarkable protective effect and a combination supplementation therapy involving melatonin and other antioxidant vitamins could be more effective in potentiating the protective effect of melatonin on metal toxicity especially toward the toxicity induced by metallic mixtures.