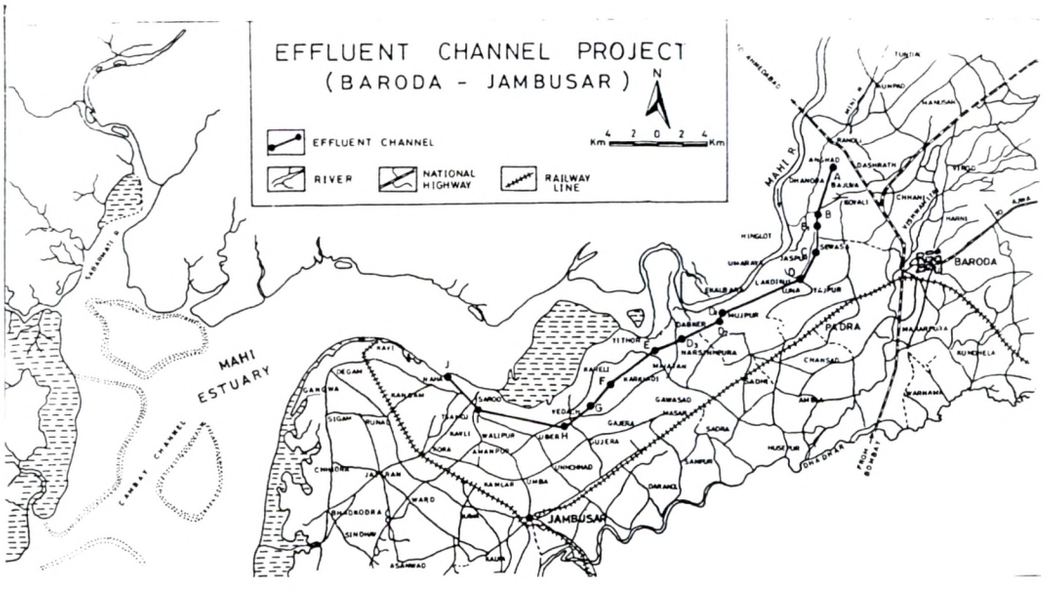
INTRODUCTION

A peaceful, serene and pollution free environment is a prerequisite for the healthy living of human being. The environment is the sum total of surroundings of man which includes social, biological economical, and physico-chemical factors and, a delicate equilibrium between human being and its niche is indispensible for the proper functioning of human body including normal growth and development. Unfortunately, in the race for industrialization, his greediness to over exploit natural resources and his meaningless attempts to conquer nature for past couple centuries have resulted in creating a hostile environment for himself and other living beings, posting a big question about the quality of existence in our blue planet. Industrialization, without any foresight, is introducing a number of toxicants into the environment in the form of industrial waste, and automobile exhaust resulting in contaminated food, water, air and soil. Vadodara is also a victim of such a thrust for industrialization, a race which is making Vadodara population vulnerable to toxicant exposure.

Vadodara, formerly known as Baroda, a cultural capital of Gujarat, is a landmark spot in the chemical industry map of India; it is surrounded by three GIDCs and mammoth sized industrial plants like GSFC, Refinery, Heavy Water Plant, IPCL, etc. Apart from these industrial giants, there are number of other pharmaceutical industries, paint and pigments industries, dry cell battery factories, dye making units,

and many more small scale units housed under GIDCs. These industries release a wide spectrum of chemical effluents into the Baroda effluent channel. These industrial wastes contain various hazardous organic and inorganic chemicals including heavy metals. This effluent channel is 56 km. long and Asia's second longest channel of its kind.



Taken from Sharma Thesis, 1996

Some farmers use this contaminated water for growing variety of pulses, cereals and vegetables in their fields on either side of the effluent channel (Sharma and Ramachandran, 1995-unpublished). Use of this effluent for agricultural activities can lead to accumulation and biomagnification of the hazardous toxicants in the food

crops. As a result, the heavy metal content in the vegetables and cereal grains grown in and around Vadodara is much higher than the permissible limit. Past data from our laboratory has shown the heavy metal content in these cereals and vegetables to be in the range of 3-20 times higher than what the World Health Organization recommended. Moreover, the previous studies suggest that the concentration of chromium, cadmium and nickel to be predominant in the food crops grown in and around Vadodara to which its residing population is exposed to.

Chromium(VI):

Group VI of the periodic table has transition metals, one of which is Chromium. It was discovered by Vanquelin in 1797 and is also an essential trace element of human nutrition. The outer electronic configuration of Chromium is $3d^5$ $4s^1$ and able to form compounds having oxidation states Cr(II-) to Cr(VI+). Cr(VI) and Cr(III) oxidation states are most common and stable states found in nature. Cr(VI), a major form of chromium pollutant is present in the human environ due to anthropogenic activities and its extensive usage in more than 50 different industries such as welding, chromate production, chrome plating, cement, rubber, ferrochrome, battery, pigments candle, paints, etc. (Barceloux, 1999; ASTDR, 2000).

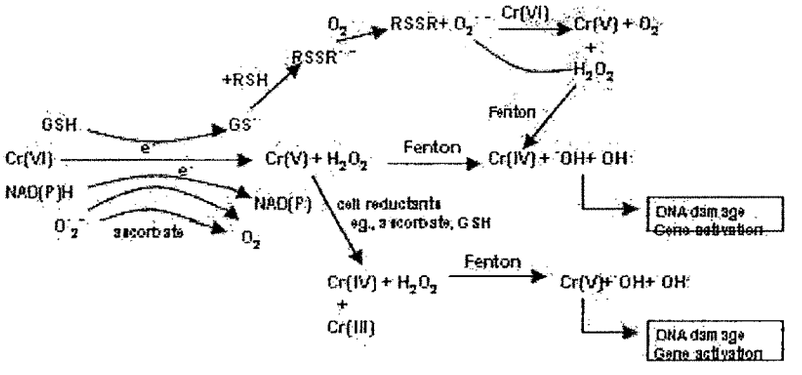
Chromium(III) is an essential trace element, and its daily human dietary requirement ranges from 4 - 30μg (Mertz, 1991) and, influences glucose and fat metabolism.(Anderson *et al.*, 1997). Some of the Cr(III) compounds like niacin-bound chromium(III), which is also known as chromium polynicotinate, chromium chloride and chromium picolinate are usually given as dietary supplements (Bagchi *et al.*,

2002). Chromium is also involved in hepatic cholesterol biosynthesis (Curran, 1954). Low-molecular-weight Cr-binding substance (LMWCr), also called as *chromodulin*, is a biologically active form of Chromium. This 1500 Da mammalian polypeptide is able to bind four trivalent chromium ions and able to enhance the insulin signaling pathway (Vincent, 2000; Davis *et al.*, 1996).

Humans are vulnerable to Cr entry in their body mainly either through occupational exposure or non-occupational exposure. Chromium species make their entry via inhalation or through skin in workers due to their occupational compulsion whereas, general human population is exposed to Cr through food and water (Landgard, 1982; Pedersen, 1982, Hertel, 1986). Morris *et al.* (1990) reported absorption of Cr(VI) by lungs and GI tract. Studies have revealed the amount of accumulation of Cr in various organs in rats to be in the following order; liver > kidney > testis > brain > blood (Tandon *et al.*, 1979). Following their entry into the animal body, hexavalent chromium can enter the cell via SO_4^{2-} and HPO_4^{2-} channels (Codd *et al.*, 2001) but not trivalent chromium and hence considered relatively non-toxic and safe (Barceloux, 1999).

After reaching inside the cell, Cr(VI) gets reduced to stable oxidation state *i.e.* Cr(III); during this reaction two intermediate oxidation states (Cr(V) and Cr(VI)) of chromium are formed (Connett and Wetterhahn, 1985; DeFlora, *et al.*, 1990). Oral administration (through drinking water) of hexavalent chromium has been known to generate ROS and alter antioxidant status in testis and epididymis (Subramanian *et al.*, 1999; Aruldhas *et al.*, 2005). A schematic diagram depicting role of various

biological reductants and various reactions involved in the reduction of Cr(VI) is given below.



Taken from Valko *et al.*, 2005 → Fig.: 3 pp: 1178

Literature survey reveals various toxicity manifestations like neurotoxicity, hepatotoxicity, dermatotoxicity, developmental toxicity, renal toxicity, reproductive toxicity, genotoxicity and cytotoxicity of hexavalent chromium (Diaz-Mayans *et al.*, 1986; Sarkar *et al.*, 1993; Junaid *et al.*, 1996; Kanojia *et al.*, 1996, 1998; Samitz, 1970; Bagchi *et al.*, 2002; Aruldhas *et al.*, 2005). Both human and animal studies have confirmed chromium species such as calcium chromate, zinc chromate, lead chromate, strontium chromate, sodium dichromate and chromium trioxide to be potent carcinogens (IARC, 1990).

Sikka (1999) suggested that testis is more sensitive to toxic effect of xenobiotic than other organs of male reproductive system. Chromium can disrupt the blood testis barrier (BTB) (Pereira *et al.*, 2002). Interestingly, Cr(VI) treatment in rats

can decrease leydig cell number and inhibit of 3β-HSD activity leading to marked decrement in serum testosterone titre. A study conducted on male New Zealand white rabbits reported decreased sperm count and motility and increased sperm abnormality and mortality (Yousef *et al.*, 2006). Above findings strongly suggest that chromium(VI) exposure may cause steroidogenic and spermatogenic impairments leading to male infertility.

Although there is adequate literature available regarding the hexavalant chromium induced testicular toxicity, data regarding the effect of Cr(VI) induced epididymal dysfunction are very less. Since, epididymis is an important male reproductive organ involved in sperm maturation, it warrants a through investigation on toxic manifestations of Chromium. Moreover, the effect of realistic (environmentally simulatable) dose of oral exposure of Cr(VI) for chronic treatment is lacking. Thus, the effect of chronic oral exposure of Cr(VI) on male reproductive organs needs to be evaluated in terms of alterations in oxidative stress steroidogenesis, spermatogenesis and sperm features.

Cadmium:

The word cadmium was derived from the Latin, (cadmia) and Greek (calamine) literature meaning cadmium bearing mixture of metals which was named after the Greek mythological character Cadmus. Cadmium was discovered in 1817 by Friedrich Strohmeyer in Germany. Atomic number of cadmium is 48 and is a transition metal present in group 12 of the periodic table, having most common oxidation state Cd^{+2}, however rarely found as Cd^{+1} (Valko *et al.*, 2005). Cadmium is

having diverse applications in industry and out of 100% usage of Cd in the industry, around 82% is used for the production of Ni-Cd dry cell batteries. The other major applications of this element are in pigments, coatings, stabilizers and alloy industry.

There is no biological role of Cd reported except for the recently reported Cd dependent carbonic anhydrase in marine diatoms (Lane *et al.*, 2005). Further, ATSDR (Agency for Toxic Substances and Disease Registry, 1999) has ranked cadmium as the 7th most toxic compound in its priority list. World wide release of Cd due to anthropogenic activity is ten times higher than the natural one which leads to contamination of air, water, food and soil. Humans are vulnerable to Cd intoxication through water and/ or food they consume, mainly in the form of leafy vegetables and meat of the animals including fishes (IARC, 1993). Primary route of Cd entry into human body is through contaminated food or water; though cadmium is very poorly absorbed by the intestine (~5-10%). Cadmium is present in the free ionic form in water and dietary components and is present as a Cd ion bound to ligand proteins like metallothionein (Nordber *et al.*, 1986; Crews *et al.*, 1989; Groten *et al.*, 1990). Following its entry into the animal body, Cd rapidly invades the body due to its high volume of distribution (Monsefi *et al.*, 2009). Accumulation of Cd is confirmed by various workers in kidney, liver, lungs, brain, testes and male reproductive tract (Danielsson *et al.*, 1984; Oldereid, 1993; Kusakabe *et al.*, 2007; Monsefi *et al.*, 2009). The severity of the cadmium toxicity is due to its longer biological half life (15-30 years in human and 6 months in rats) and very low rate of excretion which allows cadmium to stay in soft tissues for long duration making it more hazardous to humans and animals (Yan *et al.*, 2003; Benoff *et al.*, 2008).

Though the male reproductive toxicity of cadmium was reported way back (Alsber and Schwarze, 1919), Cd toxicity came into limelight at around 1946 due to the identification of the causative agent, *i.e.* cadmium, of "*itai itai beyo*" (*itai itai* meaning ouch ouch and *beyo* meaning disease) in Japan. Due to extensive mining around the area of Jinzu river of Japan, Cd used to contaminate the water of river which was mainly used by the local population for drinking and irrigation purposes leading to its accumulation in the rice field and subsequently making its entry into the body of local population and caused brittleness of bones (osteomalacia) and kidney failure. This event ignited a great concern and research about Cd toxicity.

Cadmium is not able to generate ROS by itself but can contribute to significant amount of oxidative stress of tissues due to indirect production hydroxyl and nitric oxide (Galan *et al.*, 2001). However, recent study using *in vivo* spin trapping technique has established that Cd can generate reactive oxygen- and carbon-centered radical species in rats (Liu *et al.*, 2008). Deleterious effect of Cd on various organ system is well documented (ATSDR, 1999). Cadmium can exert respiratory toxicity (Petering *et al.*, 1979), GI tract irritation (Machemer and Lork, 1981), hepatic toxicity(Andersen *et al.*, 1988; Sidhu *et al.*, 1993), cardiac damage (Schroeder *et al.*, 1965; Jamall *et al.*, 1989), hematological effects (Masaoka *et al.*, 1994), renal damage (Buchet *et al.*, 1990) and endocrine disruption (Wilson *et al.*, 1941). Literature survey also suggests that Cd exposure can cause testicular damage, altered steroidogenesis and hampered spermatogenesis leading to male infertility in different animal models such as guinea pigs, dogs, mice, hamsters, rats, etc. (Allanson and Deanesly, 1962; Johnson, 1970; Nordberg, 1971; Aoki and Hoffer, 1978; Hew *et al.*, 1993; Xu, 1996)

but, literature related to effect of Cd on epididymis is very sparse (Sacerdote *et al.*, 2008).

Nickel:

Nickel – the 28th element of periodic table was discovered by Axel Fredrik Cronstedt in 1751 is having oxidation states from -1 to +4; however the predominant oxidation state is +2. Nickel is an essential element and plays an important role in hemopoiesis by facilitating the absorption of ferric ion from intestine (Nielsen *et al.*, 1984). Due to wider industrial application of Ni such as electroplating, polishing, production of Ni-Cd batteries, refining, food processing, *etc.* It is difficult to avoid presence of Ni in human environs (Das and Dasgupta, 2002). Non-occupational exposure of Ni to general population is via contaminated water, food, air, dermal contact (jewelry and artificial body parts), and smoking (Das and Buchner, 2007). Non-smoking and non-occupationally exposed human beings are at the risk of Ni intoxication primarily through dietary sources. Once internalized into the animal body, nickel is transported by albumin and nickeloplasmin (Tanaka *et al.*, 1985) and can enter cells either by simple diffusion, if the Ni species is fat soluble, or through ionomycin, ionophore calcium channel and, insoluble nickel species enter the cell by phagocytosis (Costa *et al.*, 1982; Refvik and Andreaseen, 1995).

Though this metal gets poorly absorbed in the intestine (~1-2%), it can penetrate into the soft (liver, kidney, etc.) and tough (bone) tissues of the body (ATSDR, 2003). Ni is known to cause various deleterious effects including carcinogenic and mutagenic effects as studied in different animal models and human

(Kakela *et al.*, 1999). Further, Ni is also known to alter the functional ability of many organs such as kidney, liver, lungs, and testes and even after metabolism of ascorbic acid and cholesterol (Das Gupta *et al.*, 2008). The toxicity of nickel may be due to its capacity to generate low but measurable amount of free radicals in the cell leading to increased oxidative stress and lipid peroxidation and decreased activity of antioxidant defense (Dally and Hartwig, 1997).

Nickel administration has recently been shown to increase testicular oxidative stress and decrease the activity of antioxidants leading to DNA damage (Doreswamy *et al.*, 2004). Nickel compounds are also known to inhibit the activity of steroidogenic enzymes, 3 β – HSD and 17 β - HSD, which leads to altered steroidogenesis resulting in lower testosterone production (Das and Dasgupta, 2002). Apart from the above mentioned studies, literature survey reveals that there are some reports available on nickel induced male reproductive toxicity though, majority of these studies are restricted to testicular functions alone and epididymis, despite its importance in sperm maturation, is not given much attention. Moreover, majority of the available reports show the employment of single and/ or low dose of Ni which are not physiologically relevant to humans and, data pertaining to male reproductive effects of chronic administration of Ni through natural route of exposure is lacking.

Melatonin:

Recent advancement in the field of electronics has given chance to wrist watch manufacturer to include number of functions such as calendar, radio, mobile phone, stopwatch, alarm, timer, television, *etc.* in it with which, one can explore different

functions anywhere and anytime as per one's requirements. One such versatile indolamine present ubiquitously in nature and thought to be the phylogenetically oldest biological signaling molecule is Melatonin. Apart from its clock and calendar functions (*i.e.* signaling the 'time of day'-circardian and time of 'time of year'-circannual), due to which this indole is considered as chronobiological pacemaker or internal synchronizer ("Zeitgeber") (Cardinali *et al.*, 1997), melatonin can also influence diverse physiological events such as puberty, seasonal sexual activity cycle, prevent aging, boost immune functions, *etc.* (Andreeva *et al.*, 1999). Though, among vertebrates, melatonin is primarily secreted by the pineal gland, it is also synthesized by various other organs such as bone marrow cells (Conti *et al.*, 2000), skin (Slominski *et al.*, 2005), retina (Cardinali and Rosner, 1971; Tosini and Menaker, 1998; Liu *et al.*, 2004), GI tract (Bubenik, 2002), platelets (Champier *et al.*, 1997) and testis (Tijmes *et al.*, 1996) etc.

This tryptophan derivative, having molecular weight of 232.278 g/mol, was extracted from the mammalian pineal gland and chemically characterized by Larner and co-workers (1959). Axelrod (1974) for the first time explained the enzymes involved in melatonin biosynthesis in pinealocytes. Tryptophan is converted to serotonin via intermediary product 5-hydroxytryptophan. Acetylation of serotonin by rate limiting enzyme AA-NAT (arylakylamine N-acetyltransferase) results in the formation of N-Acetylserotonin which is subsequently converted to melatonin by HIOMT (hydroxyindole *O*-methylatransfersase). In mammals, production of melatonin is under the control of suprachiasmatic nucleus (SCN) in the hypothallamus of brain and is influenced by the presence of environmental light (Moore, 1997). Pineal melatonin release shows a circardian rhythm as its level during day (photophase) is low and at

night(scotophase) is high. Majority of the vertebrates follow such pattern of melatonin release irrespective of their habit *i.e.* nocturnal or diurnal (Claustrat *et al.*, 2005). After its release, melatonin completes its entry in all tissues within a very short period of time (Cardinali and Pevet, 1998; Macchi and Bruce, 2004). Half life of melatonin is 2 – 20 minutes and follows a bi-exponential pattern; into a first distribution half life of 2 minutes and second distribution half life of 20 minutes (Claustrat *et al.*, 2005).

Though melatonin present in the blood circulation is metabolized in liver and extrahepatic tissues to 6-sulfatoxymelatonin (aMT6S) and 5-methoxytryptamine, it can also be metabolized by non-enzymatic kynurenic pathway in all cells and extracellular matrix by free radicals and other oxidants into AFMK (N^1-Acetyl-N^2-formyl-5-methoxykynuramine) and AMK(N^1-Acetyl-5-methoxy-kynuramine).

Interestingly, melatonin diffuses very easily through biological membranes and hence can influence almost all body cells. Concurrently, the effects exerted on the cells may be receptor mediated or receptor independent. Membrane receptors (MT_1 and MT_2) of melatonin are G-protein coupled and contain typical seven transmembrane domains. Both MT1 and MT2 receptors are involved in chronobiological at the level of effect at SCN where, MT1 suppress neuronal firing activity and MT2 induces the phase shift (Dubcovich and Markowska, 2005). Apart from the membrane receptor, melatonin also binds to nuclear receptors of retinoic acid super family RORα1, RORα2 and RZRβ. RORα1 and RORα2 and are thought be responsible for immune modulation, RZRβ is expressed in pineal gland and central nervous system (Wiesenberg *et al.*, 1995; Carlberg, 2000).

Presence of melatonin in almost all taxa of living organism including bacteria, algae, plants, invertebrates and vertebrates seems to convey a different role of melatonin other than a hormone. And indeed, the land mark experiment of Tan *et al.* (1993) confirmed the role of melatonin as an antioxidant and free radical scavanger. Almost after four decades since the discovery of this indolamine, only in the last decade of the 20^{th} century, the role of melatonin as a free radical scavenger was established. Following the encouraging results of Tan *et al.* (1993), other workers including him within three years through a series of experiments provided evidences for the melatonin is an efficacious free radical scavenger, powerful antioxidant and for its protective role against lipid peroxidation caused due to attack of free radicals on the biological membranes (Tan *et al.*, 1993; Pieri *et al.*, 1994; Melchiorri *et al.*, 1996). Subsequently, scientists gauged the capacity of antioxidants to scavenge notorious $ROO^{\bullet}$ in the following order melatonin > trolox > ascorbic acid > GSH (Pieri *et al.*, 1995). Biological membranes are also more susceptible to the devastating actions of hydroxyl radical, and in this context, property of melatonin as a most potent hydroxyl radical scavenger affords marked against damage of biological membranes (Hardeland *et al.*, 1993; Sonmez *et al.*, 2007). Further, the popularity of melatonin as a powerful scavenger of hydroxyl radical is due to the fact that melatonin metabolites (AFMK and AMK) formed via kynurenic pathway also protect non-hepatic tissues against high concentration of this radical (Tan *et al.*, 2003).

Melatonin at pharmacological and probably even at physiological levels increases the activity or stimulates gene expressions of enzymatic antioxidants such as SOD (both Mn-SOD and Cu-Zn-SOD), GPx, G6PD and GR (Pablos *et al.*, 1995; Pierrefiche *et al.*, 1995; Antolin *et al.*, 1996; Pablos *et al.*, 1997). These enzymes are

highly important for the survival of a cell as they exert marked protection against oxidative stress and their increased activity help cells to successfully nullify the free radicals. Interestingly, melatonin offers double protection one by enhancing the activity of enzymatic antioxidant and secondly, by inhibiting the activity of pro-oxidative enzyme such as nitric oxide synthase (a calmodulin dependant pro-oxidative enzyme) by binding to calmodulin (Pozo *et al.*, 1997). Moreover, melatonin when being internalized into the cell membranes occupies the position near the polar head of phospholipids and protects the biological membranes by directly scavenging free radicals and thereby aids in stabilizing and maintaining functional efficiency; celluar membranes, this salient feature of natures most efficacious indole further contributes to its antioxidative potency.

Adequate data is available regarding the influence on melatonin in regulating reproductive functions in seasonally breading mammals. These effects are mainly exerted due to the inhibitory actions of melatonin at various check points of HPG axis (hypothalamic-pituitary-gonadal axis). In general, it is believed that melatonin act as an antigonadotropic agent due to its inhibitory effect at the level of hypothalamus and pituitary (Jackson *et al.*, 1982; Bittman *et al.*, 1985). It has also a direct inhibitory action on testicular steroidogenesis following gonadotropin stimulation (Tijmes *et al.*, 1996). Moreover, its protective effect on xenobiotic compound induced oxidative stress in reproductive organs, is less documented in the literature (Rao and Gangadharan, 2008).

Progressively declining semen quality and quantity of human males are of great concern in fertility and reproduction research (WHO, 1999). This decline in

semen quality and quantity is due to the increased incidence of exposure of male reproductive organs to xenobiotic compounds including heavy metals, due to occupational exposure or environmental exposure including dietary sources leading to markedly increased oxidative stress. In this context, administration of a powerful antioxidant can protect male gonads from the oxidative stress mediated deleterious structural and functional changes and conserve the structural integrity and functional capability of male reproductive organs which are prerequisites for production and maturation of male gametes.

Objective:

Classical metal toxicity studies are based on LD_{50} values. In natural environment, we are never exposed to a classical toxicological dosage. Going through literature, a good amount of information is available for single metal studies but hardly any study on multiple metal toxicity is available. Majority of the available reports are again for single dose exposures or even if multiple exposures are used, they are for shorter duration of metal treatment only. Most notable observation about the route of administration in the reported data is that, numerous studies have employed intraperitoneal, intradermal, intramuscular, subcutaneous and intravenous modes of administration, which are not the actual mode of entry of toxicants including heavy metals into our body. Many studies have tried to assess the protective roles of vitamins and synthetic analogs and have got positive results, however, many of these synthetic compounds and vitamins when administered for longer duration at higher dosage exerts toxic manifestations.

Considering all the shortcomings of available literature and presence of high content of metals (mainly Cr, Cd and Ni) in the dietary components to which Vadodara population is exposed, the present study was designed to evaluate male reproductive toxicity of hexavalant chromium, cadmium and nickel, either singly or in combination provided through drinking water on *wistar* rats for three different durations (15, 30 and 60 days).

Accepting the bitter fact that, entry of toxicants including metals into the human body is unavoidable due to food habits and life style, the possible use of efficacious natural antioxidants which can counter the ill effects of food such as metals is the need of the hour. One such natural, efficacious, and non-toxic antioxidant, Melatonin has been tested as a possible therapeutic agent to protect against metal (single or multiple) induced insult of male reproductive organs (testis and epididymis).