CHAPTER IV
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STUDIES ON THE EFFECTS OF ALUMINIUM INGESTION TO ADULT MALE ALBINO MICE (30 AND 60 DAYS)

During the tenure of the present investigation, the toxic effects of aluminium chloride ingestion for varied durations (30 and 60 days) was studied in order to evaluate time related changes on the structure and physiology of reproductive and other organs and fertility of adult male mice (Mus musculus) of Swiss strain.

Aluminium chloride (AlCl₃) was administered at a dose of 200 mg/kg. body weight/day for 30 and 60 days. The dose was selected based on the LD₅₀ value of aluminium in male mice which was experimentally found in our laboratory to be 4g/kg body weight (Chinoy and Bhattacharya, 1997).

The treatment was administered orally, since the main sources of intake of aluminium is through foods, beverages, pharmaceutical products and leaching from utensils. The durations of the study were 30 and 60 days as one spermatogenic cycle requires a period of 30 to 32 days in mouse and the entire spermatogenic process as well as sperm maturation period in the epididymis is completed by 45-50 days.
The parameters studied at the end of the treatment were sperm motility of the cauda epididymal spermatozoa, their live: dead ratio, fertility rate as well as histology and ultrastructure study of the reproductive organs mainly testis, epididymis and vas deferens.

In addition, the levels of protein in different organs viz., testis, cauda epididymis, vas deferens, muscle, liver and kidney were also investigated. The concentration of glycogen and activity of phosphorylase in the vas deferens, levels of blood glucose and fructose in seminal vesicle were studied to investigate the alterations in carbohydrate metabolism, if any. Similarly, certain specific androgen dependent parameters in target organs were also investigated viz., cholesterol, activities of 3β and 17β hydroxysteroid dehydrogenase in testis, and serum testosterone levels by RIA were investigated, to study the impact of AlCl₃ on testicular functions. Further, succinate dehydrogenase (SDH) in testis and cauda epididymis, adenosine triphosphatase (ATPase) and sialic acid in cauda epididymis, were also investigated.

The levels of DNA, RNA in testis, cauda epididymis and muscle using human lymphocyte culture and by incorporating different doses AlCl₃ (100, 200, 300 mg/ml) in the medium and chromosomal aberrations were evaluated to investigate the effects of aluminium on nucleic acid metabolism and genotoxicity. Concentrations of Na⁺, K⁺, in kidney and the tissue burden of aluminium in testis, cauda epididymis, vas deferens, kidney and serum were also studied. Apart from this, the levels of serum glutamate
oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) which are considered as specific markers of liver functions were also investigated.

The aluminium levels in several tissues were determined to study the effect of tissue burden.

The possible reversibility of induced effects were investigated by withdrawing the treatment for 30 and 60 days after administration of AlCl₃ for 30 days at a dose of 200 mg/kg body weight. In a different set of experiments, the treatment was withdrawn for 60 days after 60 days of AlCl₃ ingestion.

In view of aluminium induced toxic effects, the therapeutic action of some agents viz., calcium and vitamins (C, D and E), and their ameliorative role was also explored.

**HISTOLOGY**

The histological studies on testis, cauda epididymis and vas deferens of male mouse treated with 200 mg/kg body weight AlCl₃ revealed structural alterations after 30 days in all the organs investigated. AlCl₃ brought about disorganisation of the germinal epithelium with denudation of the germinal cells and pyknosis of germinal cell nuclei was observed in testis. Arrest of spermatogenesis was also observed in the tubules, where the lumen had scanty sperm bundles. Similar necrotic changes in the testis of mice treated
with aluminium nitrate at the doses of 100 and 200 mg/kg/day for 4 weeks were reported by Llobet et al. (1995). They observed necrosis of spermatocytes / spermatids in five and six mice, respectively, out of ten mice from each dose group. Leydig cell vacuolization was observed only at 100 mg/kg/day group, whereas, the tubular diameters were unaffected by treatment with aluminium nitrate (Llobet et al., 1995).

In the present study the cauda epididymis tubules showed degeneration and vacuolization in the epithelial cells with pyknosis of their nuclei and loss of stereocilia. The spermatozoa bundles in the tubular lumen appeared clumped. The vas deferens of animals treated with aluminium also showed decrease in muscle layers and absence of stereocilia.

The ultrastructural studies using an EM900 Zeiss Electron microscope also revealed alterations in the testis, cauda epididymis and vas deferens of treated mice. The ultra structure of the testis was most severely affected. The above mentioned alterations in the histology and the ultrastructure of these organs might be related to the tissue burden of the aluminium.

In the present study, the analysis of aluminium levels in testis, cauda epididymis, vas deferens, kidney and serum of AlCl₃ treated mice revealed significant enhancement, which indicates that the aluminium accumulates in these tissues and would affect their structure and metabolism. It is known that all absorbed aluminium is not excreted in the urine and some amount
accumulates in tissues. The accumulation of aluminium in tissues occurs when humans and animals have high concentrations of aluminium in their sera which reflects the inability of the kidneys to readily excrete aluminium (Greger, 1993). In the present study to kidney malfunction might be the causative factor for increased tissue burden of aluminium. A similar accumulation of aluminium in various tissues of different animals has been reported after exposures for different durations (WHO, 1997).

Aluminium Chloride treatment caused a significant decrease in protein levels in testis, cauda epididymis, vas deferens, muscle, liver and kidney which might be due to changes in its synthesis and/or metabolism. The decline observed in protein levels in various organs in the present study would reduce their secretions and affect the activities of various enzymes. These results might be related to the changes in histology and accumulation of aluminium in these organs viz., testis, cauda epididymis, vas deferens and kidney. Aluminium accumulation also occurred in liver of aluminium treated animals (Van der Voet, 1992). Though, very little information is available on aluminium metabolism in muscle, it is suggested that muscle could be loaded with aluminium after treatment. Thus, it is evident that tissue burden of aluminium causes disturbances in protein synthesis.

The succinate dehydrogenase (SDH) is an oxidative enzyme involved in the Krebs cycle. In the present study, a significant decrease was observed in the activity of SDH in testis, cauda epididymis and gastrocnemius muscle of aluminium treated mice. This would affect the conversion of succinate to
fumarate and may cause a block in the Krebs cycle. Moreover, SDH is a mitochondrial enzyme and its decreased activity indicates a possible alteration in mitochondrial structure and function as a result of aluminium ingestion, probably related to the disruption of mitochondria and their cristae in testis and cauda epididymis of aluminium treated mice. Chinoy and Bhattacharya (1996, 1997) had reported a decrease in SDH activity in testis and cauda epididymis of aluminium chloride treated mice.

The study conducted by Jagannatha Rao (1992), on the effect of aluminium on the brain cells of the rat revealed inhibition of membrane-bound Na\(^+\), K\(^+\), ATPase activity. An \textit{in vitro} kinetic study by Jagannatha Rao (1990) on the effects of aluminium salts on synaptosomal key enzymes, ATPase (Na\(^+\) - K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) dependent), found a significant inhibition of this enzyme activity, among which kinetics of Ca\(^{2+}\) - ATPase inhibition by AlF\(_3\) was found to be non-competitive.

In the present study, the ATPase activity showed a significant decline in cauda epididymis and muscle in treated mice. ATPase plays a role in sperm motility and metabolism and energy supply. The restricted energy supply for the sperm as a result of decrease in ATPase would affect sperm motility. In the present study, the sperm motility was significantly reduced after treatment. A decrease in muscle ATPase would also affect its contractility.
The intracellular Na\(^+\) - K\(^+\) balance is necessary for ATPase activity. In the present study the Na\(^+\) levels were significantly decreased while K\(^+\) were not much affected in treated mice. This might inhibit Na\(^+\) / K\(^+\) dependent ATPase activity (Brooks, 1984). Thus, decreased ATPase activity would be responsible for decline in sperm motility and consequently fertility rate. Sialic acid, a sialomucoprotein, is essential for sperm maturation and the maintenance of structural integrity of its membranes (Prasad and Rajalakshmi, 1976; 1977). In the present study the levels of sialic acid showed an insignificant decline by aluminium treatment which would also contribute to the alterations in sperm maturation and its structure as revealed by ultrastructure studies.

**EFFECTS OF ALUMINIUM CHLORIDE ON CARBOHYDRATE METABOLISM**

De Bruin (1976) reviewed the effects of aluminium on carbohydrate metabolism. Aluminium salt when ingested at toxic levels caused profound disorders in phosphate metabolism. These abnormalities were accompanied by significant decrease in hepatic and muscle glycogen levels. Aluminium intoxication is also known to produce hyperglycaemia.

The 30 and 60 days AlCl\(_3\) administration caused a significant enhancement in the levels of glycogen in vas deferens, muscle, liver and kidney. The elevated glycogen levels in these tissues was accompanied by a significant decrease in the activity of phosphorylase. This might be the main causative factor which might have led to the accumulation of glycogen. As a
result of decreased glycogen utilization, a significant decline was obtained in the blood glucose levels of aluminium chloride treated mice. Similar results have been reported by fluoride ingestion in mice (Chinoy et al., 1994a, b; Chinoy and Patel 1996; Patel and Chinoy, 1997).

Fructose also plays an important role in providing energy to the sperm. However, the levels of fructose did not change in seminal vesicle after treatment which suggests that its metabolism was not affected by the treatment as for glycogen.

**EFFECTS OF ALUMINIUM ON STEROIDOGENESIS**

In the present study, the exploration of intermediary enzymes in the steroidogenic pathway after aluminium treatment revealed a significant decline in the activities of 3β hydroxysteroid dehydrogenase (HSD) (which converts dehydroepiandrosterone into androstenedione), a less decline in the activity of 17β hydroxysteroid dehydrogenase (which converts testosterone to androstenedione). These results were correlated with an accumulation of cholesterol in the testis.

In the present study the histology and ultrastructure of the testis revealed structural alterations in the epithelial cells and Leydig cells of aluminium treated mice. This might be one of the causative factors for the accumulation of cholesterol. However, the testosterone levels were not significantly altered after treatment with aluminium.
EFFECT OF ALUMINIUM ON NUCLEIC ACIDS, SISTER CHROMATID EXCHANGE (SCE) AND CHROMOSOMAL ABERRATIONS

Jagannatha Rao et al. (1993) found that aluminium has no influence on mobility of linear DNA in agarose gels but it relaxed the intact supercoiled state of pUC18 DNA. Another study on the effect of aluminium on calf thymus DNA by Jagannatha Rao and Divakar (1993) indicated that aluminium binding induces numerous reversible changes in DNA structure.

In the present data, an increase was observed in DNA and RNA levels in testis, cauda epididymis and muscle of aluminium treated mice. This might be due to alterations/blockage in the synthesis of DNA and RNA. Several studies have demonstrated that aluminium inhibits RNA synthesis and hence could have an effect on its levels (WHO, 1997). The enzymes responsible for DNA and RNA synthesis on repair need to be studied in the future.

Chromosomal aberrations and sister chromatid exchanges (SCE) which are believed to be caused by strand breakage resulting in apparently homologous strand interchange and reunion during DNA replication, revealed an increase in the study group, as compared to control. However, Leonard and Gerber (1988), did not find any carcinogenic, mutagenic or teratogenic effects in in vivo and in vitro studies, except, in cases of extremely high exposures.
EFFECT OF ALUMINIUM ON SPERM METABOLISM AND FERTILITY RATE

The sperm motility and metabolism were affected by AlCl₃ treatment which would ultimately influence their fertilizability. The results of the present study elucidate that aluminium has an important role in male reproduction which revealed a significant decrease in cauda epididymal sperm motility, cauda epididymal live : dead ratio and fertility rate. The implantation sites in females mated with treated males were also significantly decreased.

Aluminium chloride has been reported to inhibit the sperm entry into the cervical mucus at different concentrations (10 to 200 μM/ml). Llobet et al. (1995) found decreased pregnancy rate in the females mated with males previously treated with 100 or 200 mg/kg/day of aluminium nitrate. High dose to male mice (200 mg/kg/day) caused significantly decreased testicular and epididymal weights, as well as in testicular spermatid counts and epididymal sperm counts. However, the sperm motility was unaffected in their study. This discrepancy might be due to the differences in the aluminium salts used (aluminium nitrate and aluminium chloride), mode of treatment i.e. intraperitoneal vs. oral, duration as well as the doses.

EFFECT OF ALUMINIUM CHLORIDE ON LIVER

The liver is often the target organ for a number of reasons. Most toxicants enter the body via the gastrointestinal tract, and after absorption they are carried by the hepatic portal vein to the liver. The liver has a high
concentration of binding sites. Aluminium induced damage of the lysosomes in the liver was studied by Stein et al. (1987). Increased biliary transferrin excretion following parenteral aluminium administration to rats was shown by Klein et al. (1993). Van der Voet et al. (1992) found a dose-dependent aluminium accumulation in liver and subcellular liver fractions. Aluminium overload in liver might lead to cholestasis (Klein et al. 1987), disturbance of hepatic microsomal functions (Bidlack et al., 1987; Jeffery et al., 1987) and even to induction of and binding to metal binding protein (Jeffery et al., 1987).

In the present study, the levels of serum glutamate oxaloacetate transaminase (SGOT) and serum pyruvate transaminase (SGPT) which are considered as specific markers for liver function showed significant decrease in aluminium treated animals suggesting alteration in liver structure and function. In corroboration with the above, the liver protein, glycogen, phosphorylase also revealed significant alterations in aluminium treated mice for 30 and 60 days.

**EFFECT OF ALUMINIUM CHLORIDE ON ELECTROLYTES**

As the glomerular capillaries have large pores (70 nm), substances with molecular weights under 60,000 are filtered into Bowman's capsules. Some of the filtered substances such as glucose and amino acids, which are vital to the body, are reabsorbed by the tubules. To facilitate the passive reabsorption of water and to maintain the homeostasis, various electrolytes in the glomerular filtrate are reabsorbed nearly completely or to a great
extent, the reabsorption of the Na+ at the distal and collecting tubules is regulated by the mineralocorticoids, that of phosphorus by the parathyroid hormone, and of bicarbonate (HCO$_3^-$) by the acid-base balance. In addition, K$^+$ and H$^+$ are secreted by the tubules (Lu, 1996). The present study showed a significant decrease in Na$^+$ levels, but K$^+$ levels did not reveal any change after aluminium chloride treatment in kidney. Thus, electrolyte imbalance in kidney might be due to changes in its metabolism or alterations in hormone levels. These changes in electrolytes especially Na$^+$ might affect the electrolyte balance in the blood, muscle, nerve and epididymis after aluminium exposure. Further detailed studies on these ions as well as their hormonal regulation needs to be undertaken in future.

**WITHDRAWAL STUDIES ON ALUMINIUM INDUCED TOXIC EFFECTS**

In view of the above observed aluminium induced toxic effects, in a different group of animals, AlCl$_3$ was fed for 30 days and the treatment was withdrawn afterwards for 30 and 60 days respectively (Groups X and XI). In another group of animals, AlCl$_3$ was administered for 60 days and then the treatment was withdrawn afterwards for another 60 days (Group XII). In both the cases, the dose was 200 mg/kg body weight.

During this period, the animals were maintained on standard diet and water *ad libitum*. The results revealed that in many of the parameters, recovery was only partial from aluminium induced toxicity, but not significant as compared to control. This may be due to delayed sequestration of aluminium from the body as evident by high serum aluminium levels in the
present study. However, the concentrations of glycogen in vas deferens, muscle and liver, testicular cholesterol and 3β HSD activity, DNA levels in testis and muscle, RNA levels in testis, cauda epididymis and muscle showed significant recovery after withdrawal of aluminium treatment.

**BENEFICIAL EFFECTS OF ASCORBIC ACID (AA), CALCIUM (Ca²⁺), VITAMIN D AND VITAMIN E ON ALUMINIUM INDUCED EFFECTS**

To evaluate the beneficial role of the therapeutic agents in overcoming the aluminium induced effects, different groups of animals were administered ascorbic acid (AA) (15 mg/animal/day), calcium (25 mg/animal/day), vitamin D (0.002 μg/animal/day) and E(2 mg/animal/day) alone, and in combination orally for 30 days during the AlCl₃ withdrawal period. The parameters investigated were same as those under aluminium treatment.

The results showed that administration of therapeutic agents during the AlCl₃ withdrawal period manifested significant recovery in all the parameters.

The participation of AA in cellular oxidation - reduction reactions occur via the formation of its free radical, monodehydroascorbic acid (MDHA) which is a more powerful reducing agent that AA by virtue of possessing an unpaired electron, which functions as a source of energy for sperm motility and metabolism (Chinoy, 1984) and subsequently gets oxidised to dehydroascorbic acid (DHA). DHA could be converted back to AA by
glutathione (Chinoy, 1978). Commoner et al. (1954) have correlated the concentration of free radical (FR) and biological activity in tissues. This could be the main mechanism behind the recovery observed in various aluminium induced alterations after AA treatment. Ascorbic acid is also known to bind with macromolecules like proteins, nucleic acids (Chinoy, 1978) by charge transfer complex formation, which appears to be a very active source of energy for biological processes. This seemed to be another probable mechanism occurring for the mitigation of aluminium induced toxicity. Ascorbic acid is also known to activate adenyl cyclase but inhibit phosphodiesterase (PDE) resulting in high C-AMP levels (Pasternak, 1979). The increase in C-AMP, which is known as the “Second messenger” might have resulted in the recovery in the activities of several enzymes in the present study, viz., SDH, phosphorylase, 3β, 17β HSD, etc. By virtue of being a reducing agent, AA itself is known to activate several hydroxylating enzymes in various tissues (Kutsky, 1973, Chinoy, 1978). In addition, AA treatment also brought about a significant recovery in electrolyte concentrations viz., Na⁺ and K⁺.

Calcium is known to activate several enzymes. Calcium has been found to act on beta cells of Langerhans in the pancreas and control secretion of insulin, which in turn regulates glucose levels (Rasmussen, 1989). Therefore, the recovery in carbohydrate metabolism after calcium administration could also be due to this pathway.
It is known that phosphodiesterase (PDE) catalyzes the conversion of C-AMP to 5’-AMP, thus decreasing the levels of C-AMP. However, both ascorbic acid and calcium are recognised as potent inhibitions of PDE (Pasternak, 1979). Thus, it is suggested that the increased levels of C-AMP which is important for sperm motility might also lead to recovery of many of the parameters studied. A significant regain of sperm motility and fertility by the synergistic action of AA and Ca\(^{2+}\) was also obtained.

The chief function of vitamin D is to promote the intestinal absorption of calcium and phosphorus and thus maintain an optimal blood concentration of these elements for calcification of bone. Within the kidney, Vitamin D increases the clearance of phosphate (Marks, 1975). In addition to these effects on calcium and phosphorus, vitamin D increases tissue citrate levels which acts as a chelator for aluminium. Thus, incorporation of Vitamin D might help to sequestrate aluminium from the body which might lead to recovery of all parameters studied.

Vitamin E has come under much scrutiny for its possible therapeutic roles in numerous disease states especially those involving oxidation related events (Phelps, 1987) and has proved to be the most potent biological antioxidant (Farrell, 1980; Burton et al., 1985; Burton and Ingold, 1989). Vitamin E reduces cell injury (Massey and Burton, 1989) and impedes the formation of oxidised low density lipoproteins (LDL) and their postulated atherosclerotic effects (Burton, 1990). It has also been related to changes in calcium homeostasis in the tissue (Meerson et al., 1982).
Various adverse health affects of Vitamin E deficiency in vertebrates are well documented, including disorders of the reproductive organs (Nelson, 1980). In rats, the main symptoms of Vitamin E deficiency are degeneration of the testis, abnormalities of gestation, regression in the ovary and changes in the ovulation (Marks, 1975). α-tocopherol - medicated prevention of cell injury is specially due to its maintenance of sulphydryl groups of membrane binding proteins (Jones et al., 1983). The importance of Vitamin E for protecting the integrity of lipid structures (especially membranes) in vivo is known since it is an antioxidant that has been found in plasma, red cells and tissues (Cheeseman et al., 1984).

Vitamin E has also been shown to prevent the in vivo toxicity of drugs like paracetamol in rat liver (Walker et al., 1974). Vitamin E has also been found to function as an anti-cancer agent (London, 1985). Hence, the protective effects of Vitamin E shown in this study may be of great significance in amelioration of aluminium induced toxicity. Thus, in conclusion, the aluminium induced effects could be partially reversed by withdrawal of treatment while, the subsequent supplementation of Vitamins C, D, E and calcium alone brought about more pronounced and almost complete recovery. However, the combination treatments of Vitamins D and E proved to be the most effective for recovery. Thus, aluminium induced effects are transient and reversible by the use of the above mentioned therapeutic agents. Thus, mitigation of aluminium induced toxicity could be a possibility.
STUDIES ON THE EFFECTS OF SINGLE DOSE ALUMINIUM INGESTION
TO ADULT MALE ALBINO MICE

In the course of present investigation, AlCl₃ was administered orally at a dose of 400 mg/Kg body weight as a single dose treatment. After administration, animals were kept for 15 days. During this period, they were maintained on standard diet and water given ad libitum. The parameters studied in this period, were mostly similar to those studied previously in the period of 30 and 60 days.

Aluminium decreased the levels of protein in testis, cauda epididymis, vas deferens, muscle, liver and kidney which might be due to changes in its synthesis and metabolism.

Succinate dehydrogenase (SDH), an important oxidative and androgen sensitive enzyme was significantly decreased in the testis, cauda epididymis and muscle of mice by aluminium treatment.

The depletion of ATPase in cauda epididymis and muscle and decreased sialic acid levels in cauda epididymis might be due to structural and functional alterations of these organs.

In aluminium treated mice, an accumulation of glycogen in the vas deferens was obtained which could be correlated with the decline in phosphorylase enzyme activity. Thus the rate of glycolysis would be altered. Similarly, the decrease in the levels of blood glucose would also reveal that
aluminium has an influence on carbohydrate metabolism. Fructose also plays an important role in providing energy to the sperm. However, their levels did not change in seminal vesicle which suggests that its metabolism was unaffected by the treatment.

Aluminium treatment caused an accumulation of testicular cholesterol which suggests alterations in steroidogenesis and Leydig cell functions. A reduction in the activity of $3\beta$ and $17\beta$ HSD might be one of the causative factors for effect on cholesterol metabolism and androgenesis in the testis. However, serum testosterone levels did not show any changes after aluminium treatment.

The DNA and RNA levels revealed an enhancement by AlCl$_3$ treatment in testis, cauda epididymis and muscle which might be due to effects on nucleic acid metabolism in these organs.

The discussions related to the alterations of these parameters are described in details in the previous studies of 30 and 60 days treatment.

According to Llobet et al. (1995), four weeks aluminium nitrate treated (100 and 200 mg/Kg/day) male mice showed decreased spermatid counts, epididymal sperm counts and a decline in the rate of pregnancy. In the present study too, the data revealed that aluminium chloride treatment (Single dose 400 mg/Kg body weight) resulted in alterations in the metabolism of the testis, cauda epididymis, vas deferens, seminal vesicle,
muscle, liver and kidney, thus affecting the sperm motility and fertility rate. On the contrary, Llobet et al., (1995) did not obtain any alteration in sperm motility as compared to control. This discrepancy in our data might be due to the difference in the aluminium salts used, mode of treatment, duration as well as difference in dose regimens.

WITHDRAWAL STUDIES FOR THE SINGLE DOSE TREATMENT

In a different group of animals, AlCl₃ was fed at a dose of 400 mg/Kg body weight once as a single dose treatment and then kept for 15 days. After the completion of 15 days, animals were kept for another 15 days as a period of withdrawal. During this period of 30 days (15 + 15), the animals were maintained on standard diet and water given *ad libitum*. In the withdrawal period, significant recovery was found in vas deferens and liver glycogen, phosphorylase activity in vas deferens, levels of blood glucose, muscular DNA levels and RNA levels in testis, cauda epididymis and vas deferens. Except these above data, all other parameters showed a partial recovery, as compared to control.

The above results reveal that a single large dose of aluminium chloride also causes marked toxicity in the organs studied. However, recovery occurred to a considerable extent in most of the parameters studied.