Introduction
Folates are dietary vitamins that are inevitable for cell differentiation and growth of tissue (Blakley, 1969; Davis, 1986). Folic acid is primarily reduced by an enzyme dihydrofolate reductase (DHFR) (also called folic acid reductase) to its tetrahydroform, tetrahydrofolate. This reduced form significantly performs the role of cofactor by carrying one-carbon units necessary for the biosynthesis of purine rings, pyrimidines, serine, choline, histidine, homocysteine and methionine (Stokstad and Koch, 1967; Spector et al., 1980; Zeisel, 1990). 10-Formyl tetrahydrofolate, a major folate coenzyme acts as a one carbon donor in purine synthesis (Huennekens et al., 1958). For 5,10-methylene tetrahydrofolate, tetrahydrofolate is the immediate precursor and is involved in thymidylate synthesis (Jackson and Harrap, 1973). Another major folate, 5-methyl tetrahydrofolate is known to participate in methionine biosynthesis and thus, production of S-adenosyl methionine would influence methylation process involving protein and lipid biosynthesis (Finkelstein, 1990).

In general, chemical agents that inhibit DHFR, crudely interfere with the folate metabolism. As a consequence, this sequeale causes a deficiency of one of the coenzyme forms of the vitamin that is involved as cofactors in the biosynthetic pathways of nucleic acids, lipids and proteins (Goldman and Fyfe, 1974; Erbe, 1975). The thorough understanding of folic acid metabolism paved way to recognise it as an essential factor in cellular proliferation and it became the beacon light for the development of an entire class of antifolate drugs. These drugs are used clinically as an oncolytic or antineoplastic (Pinedo, 1978; Jackson, 1984; Black and Livingston, 1990) and antimicrobial (Hitchings et al., 1966) agents.
Among the family of antimetabolites, Aminopterin and Methotrexate (MTX) or Amethopterin are the antifolates which have the nearest molecular semblance to each other (Seegar et al., 1949). One of the most important milestone in cancer chemotherapy was the discovery of aminopterin (Farber et al., 1948). Subsequently, amethopterin or MTX was synthesised and introduced into the medical armamentarium in 1949 by Seegar and his coworkers.

MTX comparatively possesses less toxic effects than aminopterin (Werkheiser, 1963). The acute LD\textsubscript{50} value for aminopterin in mice is 3 mg/kg body weight and that of MTX in rats and mice is 15 and 89 mg/kg, body weight respectively (Philips et al., 1950). Goldin (1968) reported that in experimental mouse tumor studies, MTX had a therapeutic index superior to that of aminopterin. Based on these studies, MTX supplanted aminopterin in the clinic.

1.1. Literature pertinent to MTX

*Clinical studies with high dose MTX therapy*

The ability of aminopterin to cause remission of acute lymphocytic leukemia (ALL) in children (Farber et al., 1948) is a classic observation which heralded the use of antifolate compounds in the treatment of malignancy. However, this drug is of very limited use in leukemia seen in adults (Allegra, 1990). Eight years after the report of Farber et al. (1948), the next important thumping discovery is the findings by Li et al. (1956) who demonstrated that the maximally tolerated doses of MTX produced complete recovery of choriocarcinoma and related trophoblastic tumors in
women. Cure and remedy is accomplished only in 75% of advanced cases treated sequentially with MTX and dactinomycin and in over 90%, when early diagnosis is made (Li et al., 1956). Later studies showed permanent remissions in neoplastic diseases with high dose MTX therapy (> 20 mg/M²) either alone or in combination with 6-mercaptopurine (6MP) (Acute Leukemia Group B, 1961), prednisone (Gianini and Collen, 1980; Tannenbaum, 1980), vincristine (Goldman and Fyfe, 1974), 5-fluorouracil (Bertino et al., 1977), cisplatin (Bertino et al., 1977) or with antidotes like leucovorin (LCN) (Bertino et al., 1971; Black and Livingston, 1990).

MTX therapy is also considered in the prophylaxis of graft-versus-host disease subsequent to allogenic bone marrow transplantation and also in the prophylaxis of central nervous system (CNS) in patients with acute lymphocytic leukemia (Black and Livingston, 1990). MTX is equally effective for treatment and prevention of leukemic meningitis (Allegra, 1990).

Further, MTX is successfully employed in treating gestational trophoblastic neoplasia (Bengtsson et al., 1992), hydatiform mole, chorioadenoma destruens and ectopic pregnancy (Hertz et al., 1961; Byrjalsen and Toft 1991; Crenin and Darney, 1993). MTX therapy is an alternative to surgical treatment of unruptured tubal pregnancy (Byrjalsen and Toft, 1991). MTX is successfully used in the treatment of interstitial twin pregnancy.

Apart from this, breast cancer (Greening, 1962), ovarian cancer, head, neck, bladder cancer, lymphoma (Friedman et al., 1962), soft tissue and bone sarcoma (Jolivet et al., 1983) are also highly responsive to high dose MTX therapy.
Clinical studies with low dose MTX therapy

More recently, low dose MTX therapy (7.5 - 15.0 mg/M²) has become a valuable alternative treatment for non-neoplastic diseases and has enjoyed its widest application in this respect as a treatment for severe psoriasis (Weinstein, 1977; Witty et al., 1992), Reiter's syndrome (Owen and Cohen, 1979), polymyositis (Gianini and Collen, 1980), polyarteritis nodosa (Tannenbaum, 1980), dermatomyositis (Bohan et al., 1977), mycosis fungoides (Hanno et al., 1980), Wegener's granulomatosis (Capizzi and Bertino, 1971), cyclitis (Wong and Hersh, 1965), sarcoidosis (Lacher, 1968), rheumatoid arthritis (Weinblatt et al., 1988; Bannwarth et al., 1994) and bronchial asthma (Mathur and Bhasin, 1992). However, till date, the mechanism of action of low dose MTX therapy in non-neoplastic diseases remains obscure.

In addition to this, MTX is also a potent immunosuppressive agent because of its action as an inhibitor of cell mediated immune reactions (Jackson, 1984; Chabner et al., 1985).

Combination therapy with MTX

MTX and leucovorin are given as a combination therapy in Burkitt's and other non-Hodgkin's lymphomas (Skarin et al., 1977). High dose MTX with leucovorin salvage causes remarkable tumor regression in patients with osteosarcoma (Calabresi et al., 1985). Combinations of 5-fluorouracil and MTX in advanced L1210 leukemia resulted in enhanced therapeutic effectiveness of these drugs (Bertino et al., 1977). Vincristine augments the
effect by inhibiting the efflux of MTX out of the cell, thereby increasing intracellular antifolate concentrations (Goldman and Fyfe, 1974).

**Salicylate** and **sulfisoxazole** increase free MTX levels in plasma by displacing the drug from its binding sites on plasma proteins (Mandel, 1976). **Hydrocortisone** and **prednisone** decrease the cytotoxic effect of MTX in humans (Bruckner et al., 1975).

An enhanced anticancer activity has been shown by the administration of **MTX plus 6-mercaptopurine (6MP)**. This is because MTX elevates 5-phosphoribosyl-1-pyrophosphate (PRPP) levels and results in more nucleotide trapping by 6MP in the tumor cell (Acute Leukemia group B, 1961). Zaharko et al. (1977) demonstrated increased antitumor effects after the combined administration of **MTX plus thymidine**. However, thymidine does not have the ability to save the normal tissue from drug toxicity when given alone (Tattersall et al., 1975). **MTX and cytosine arabinoside** act synergistically against childhood leukemia (Cadman and Eiferman, 1979). **MTX with sulfasalazine**, another antifolate, is effective in the treatment of rheumatoid arthritis (Tishler et al., 1988). **MTX** is also given **with parenteral gold** to treat rheumatoid arthritis (Rau, 1993).

**MTX along with azathioprine and cyclophosphamide** has been used with varying success in several diseases with immunologic reactions (Weinstein, 1977).
Fig. i: Structure of folic acid, aminopterin, methotrexate and leucovorin

Folic Acid

Aminopterin

Methotrexate

Leucovorin
MTX denotes methotrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; $\text{FH}_4$, tetrahydrofolate; $\text{FH}_2$, dihydrofolate; Glu, glutamyl; dTMP, thymidylate; and dUMP deoxy uridylate. Broken lines indicate enzyme inhibition.
Chemistry

MTX is a 4-amino-4-deoxy-N_{10}-methylpteroyl-glutamic acid (Fig.i). Its molecular structure differs from folic acid only in that folic acid has a hydroxyl group in place of the 4-amino group on the pteridine ring and there is no methyl group at the N_{10} position. MTX, also known as amethopterin, differs from aminopterin in that the latter is also not methylated at the N_{10} position (Jackson, 1984). Therefore, the active site of the MTX molecule is the pteridine ring portion (Weinblatt, 1989). MTX is a bicarboxylic weak acid with a molecular weight of 454 (Liegler et al., 1969).

Mechanism of action

MTX stoichiometrically inhibits DHFR (Osborne et al., 1958) the enzyme responsible for converting folic acid to reduced folate cofactors (Fig.ii). Reduced folates are necessary for the metabolic transfer of one carbon units in a variety of cellular biochemical reactions (Bleyer, 1978).

In thymidylate (TMP) biosynthesis, DHFR is not the rate limiting enzyme, whereas this role is accomplished by thymidylate synthase (Fig.ii), another enzyme in folate pathway (Pinedo, 1978; Chabner et al., 1986). In MTX treated cells, the rate of TMP biosynthesis shows no reduction until DHFR has become rate limiting (Goldman and Fyfe, 1974). This will take place when the enzyme activity reduced down to 95% and just 5% DHFR activity is needed to maintain adequate pools of tetrahydrofolate (Goldman and Fyfe, 1974).
The cell loses its ability to regenerate tetrahydrofolate from dihydrofolate, once DHFR has been rendered rate limiting. Under such circumstances, dihydrofolate is the product of thymidylate synthase (Goldman and Fyfe, 1974; Chabner et al., 1986). During MTX-induced inhibition, dihydrofolate accumulates in the cell and the one carbon containing tetrahydrofolate, which is the substrate to de novo synthesis of methionine, serine, thymidylate and purines gets exhausted. This results in the deficiency of purine and pyrimidine pools available for DNA synthesis (Bleyer, 1978).

MTX reduced the synthesis of DNA measured by $^3$H uridine incorporation as well as RNA synthesis during cell activation preceding S phase. Adenine appears to be the rate limiting nucleotide in purine biosynthesis, is also affected by the drug (Rosenthal et al., 1988). This inhibition of purine biosynthesis plays a major role in the suppression of humoral immunity by the antifolates (Segal et al., 1990).

Transfer of methyl group to uracil of dUMP is donated by 5,10-methenyl tetrahydrofolate and this reaction is catalysed by thymidylate synthase. This enzyme is considerably decreased after MTX treatment (Chabner and Young, 1973). Further, depletion of 10-formyl tetrahydrofolate inhibits biosynthesis of inosinic acid, a purine precursor inevitable for RNA, DNA and protein synthesis. Protein synthesis is also inhibited, as reduced folates are cofactors in the conversion of glycine to serine and homocysteine to methionine (Bleyer, 1978). Thus MTX cytotoxicity also leads to "purineless" state in cells leading to its death (Hryniuk, 1972; Allegra et al., 1986).
Homocysteine synthesised from S-adenosyl homocysteine (AdoHcy) is catalysed by AdoHcy hydrolase (Ueland, 1982). This is further converted metabolically either into cystathionine or methionine. The latter methionine pathway in tissues is catalysed by an enzyme, which requires 5-methyl tetrahydrofolate as methyl donor (Mudd and Levy, 1983). Low dose MTX therapy increases the level of homocysteine and its level is inversely related to folate status before MTX therapy (Morgan et al., 1990).

Formate gets incorporated into serine in the presence of reducing equivalents like NADPH. However, once the supply of reducing equivalents is exhausted, formate is incorporated into 5-formyl tetrahydrofolate. The reaction responsible for the formation of 5-formyl tetrahydrofolate is demonstrated to be the irreversible hydrolysis of 5,10-methenyl tetrahydrofolate by serine hydroxy methyl transferase (Stover and Schirch, 1993).

Betaine, carnitine and S-adenosyl methionine concentrations also respond to MTX administration and show a diminishing trend. (Barak et al., 1984; Pomfret et al., 1990).

There is also evidence that reduced folates are necessary for normal synthesis and metabolism of neurotransmitters of central nervous system. Any depletion in folates by MTX administration may lead to neurotoxic symptoms (Banerjee and Snyder, 1973). MTX does not interfere with the biosynthesis of prostaglandins or leukotrienes (Welles et al., 1985).
**Effect on cell cycle**

MTX acts as a poison specific for the S phase cell cycle (Skipper *et al.*, 1967). This drug is much more effective when cells are in logarithmic phase of growth, rather than in plateau phase, because MTX is capable of inhibiting RNA and protein synthesis.

**Pharmacokinetics**

MTX exerts both reversible and competitive binding with the enzyme DHFR (Bertino *et al.*, 1962). Binding of MTX to DHFR is greatly facilitated by the presence of NADPH (Jackson, 1984). Once MTX (1 μM) enters the cell, intracellular MTX is at molar equivalence with DHFR within 2 to 4 min (Gewirtz *et al.*, 1980). The complete inhibition of DNA synthesis is possible only in the presence of free intracellular drug and a proportion bound to DHFR (Goldman, 1975). MTX binding to mammalian DHFR is often 10-11M² or below and at such concentrations, there is 90% inhibition of DNA synthesis (Hryniuk *et al.*, 1975).

Fifty to 70% of the drug is bound to protein, principally albumin in human plasma (Wan *et al.*, 1974). Alterations in plasma protein binding affect the amount of free extracellular MTX, which in turn, influences the influx of MTX into cells and its rate of clearance by the kidneys (Bleyer, 1978).

**Polyglutamates of MTX** inhibit both DHFR (Galivan, 1980) and other folate dependent enzymes, such as thymidylate synthase and 5-amino-imidazole carboxamide ribonucleotide transferase (5-AICAR), enzymes
involved in the synthesis of pyrimidine and purine, which are precursors of DNA (Jackson, 1984). The intracellular binding sites of MTX and its 7-OH metabolites may well be the same (Goldman, 1975). Several low affinity binding sites have been implicated in MTX cell toxicity studies. These mainly include thymidylate synthase and serine hydroxy methyl transferase (Niethammer and Jackson, 1976).

**Distribution**

MTX distribution is not uniform and approximately 35% of the drug is bound to plasma proteins and the rest is distributed within the tissues (Huffman et al., 1973; Allegra, 1990). In conventional doses, cerebrospinal fluid (CSF) levels are only 3% of those in the systemic circulation. Hence, neoplastic cells which take refuge in the CNS are not killed by the normal dose regimen (Shapiro et al., 1975). Cytotoxic levels of MTX is achieved in CSF only at high dose therapy (Pitman and Frei, 1977).

The bioavailability of low dose oral MTX is relatively high at a range of 0.4 to 1.0 when 10 mg per M$^2$ of MTX was administered in rheumatoid patients. Intramuscular MTX is absorbed rapidly, with maximum serum concentrations attained within 2 hrs of administration (Bleyer, 1978). Plasma levels of MTX are known to peak 30-60 min after intravenous injection. MTX is distributed throughout the body with higher concentrations being found in intestinal epithelium and hepatic cells (Tishler et al., 1988). Distribution of MTX into pleural and peritoneal cavities takes place slowly. However, when these spaces expand due to effusion, they act as a site of storage and release.
of the drug, resulting in the prolonged elevation of plasma concentrations and severe toxicity (Wilkinson et al., 1978).

After intravenous administration, MTX is distributed rapidly within a volume of 18% of body weight (Leme et al., 1975) and then within a space of 76% of body weight (Henderson et al., 1965). Following intravenous injection, disappearance of MTX from plasma is triphasic (Huffman et al., 1973). The initial half-life is $0.75 \pm 0.11$ hr (Huffman et al., 1973), the second half-life has been reported to be $2.06 \pm 0.16$ hr. The terminal half-life is $10.4 \pm 1.8$ hr and it begins when the plasma antifolate concentration approaches $10^{-7}$ molar, approximately 6-24 hrs after injection of conventional doses and 30-48 hrs after high dose therapy (Stoller et al., 1975). The terminal half-life is responsible for the major portion of the bone marrow and gastrointestinal toxicity (Reich et al., 1976). These observations suggest that the toxic effect of MTX on normal tissue is more a function of the duration of exposure to suprathreshold concentrations of drug rather than the peak level achieved (Bleyer, 1978).

MTX is concentrated in the liver with liver:plasma ratios of four at 3 hrs and eight at 24 hrs after intravenous injection of 80 mg/M$^2$. (Chabner and Slavik, 1973). The drug is further concentrated in bile, with a bile (gall bladder): plasma ratio of 200:1 with MTX at 5520 mg/M$^2$ (Bleyer, 1978).

The "blood-brain barrier" slows entry of systemically administered MTX into CNS, such that under steady state conditions, the CSF:plasma ratio is 0.02-0.05 (Poplack et al., 1977).
Further, Riccardi et al. (1982) reported that in the rat, a significant blood-testis barrier to MTX exists at the tubular but probably not at the capillary-interstitial level. According to these authors, MTX levels were 2-4 fold lower in the testicular interstitial fluid and 18-50 fold lower in the seminiferous tubule as compared to plasma level.

**MTX transport**

The energetics of MTX transport are complex (Gewirtz et al., 1980). The drug enters the cell by a carrier mediated process, which is temperature, pH and energy dependent (Schilsky et al., 1981). Goldman (1973) has shown the Na⁺ dependent transport of MTX across the cell membrane. The drug uptake into various cells has revealed that its transport is mediated by two carrier systems, one with a high affinity and low capacity and the other with low affinity and high capacity (Shen and Azarnoff, 1978). Reduced folates like 5-formyl tetrahydrofolate and 5-methyl tetrahydrofolate compete with MTX for uptake and they share the same transport mechanism. However, folic acid has a unique different route for its entry into the cell (Goldman 1971; Nahas et al., 1972) as they are highly polar. They cross the blood-brain barrier very poorly, and require a specific transport mechanism to enter the cells of mammals (Goldman, 1971).

**Absorption**

In doses less than 30 mg/M², MTX is nearly completely absorbed from the gastrointestinal tract (Henderson et al., 1965). At higher doses of MTX, there is strong evidence for reduced MTX bioavailability (Smith et al., 1980).
With doses of 80 mg/M² or more, absorption from the gastrointestinal tract is protracted and incomplete, leading to plasma levels less than one-tenth that achieved after intravenous administration (Wan et al., 1974). These observations implicate the presence of a saturable intestinal absorption mechanism, possibly an active transport process, with low-capacity characteristics. In such studies, orally administered twice-weekly antifolate therapy was just as effective and just as toxic as parenteral drug (Acute Leukemia Group B, 1969).

Metabolism

MTX is metabolised during its enterohepatic circulation by bacterial degradation within the intestinal tract (Valerino et al., 1972). MTX is metabolised to (a) 7-OH MTX through the action of hepatic aldehyde oxidase (Weinblatt, 1989); (b) 2,4 diamino N-10 methyl pteroic acid (DAMPA) through the action of carboxy peptidase, an enzyme of intestinal bacterial origin and (c) polyglutamates through the action of an enzyme folypolyglutamate synthase (Hande et al., 1978; Chabner et al., 1985) (Fig.iii). These metabolites do not exhibit any tumor suppressing activity, although they may account for upto 1/3rd of the total plasma concentrations during the third stage of half-life (Huffman et al., 1973; Slordal et al., 1988). In tissues like liver and kidney, there is a prolonged entrapment of MTX for several weeks, and this retention is because of poor bypass of DHFR-mediated events by polyglutamates bound to MTX (Chabner et al., 1985). High concentration of MTX and 7-OH-MTX appear in bile within few minutes.
Fig. iii: Metabolites of methotrexate

2,4 diamino N-10 methyl pteroyl glutamate

Aldehyde oxidase

2,4 diamino N-10 methyl pteroyl poly (Y) glutamate

Polyglutamate synthetase

Carboxypeptidate

2,4 diamino N-10 methyl pteroic acid glutamic acid

7-OH methotrexate
suggesting the liver to be a major site of MTX metabolism (Bremnes et al., 1989).

**Excretion**

MTX is renally eliminated by both **glomerular filtration** and **renal tubular secretion** (Bourke et al., 1975). Under conditions of normal renal function, drug clearance from plasma in man is 110 cc/min/M², and in which about 103 cc/min/M² is due to renal clearance (Liegler et al., 1969). Approximately 41% of an intravenously administered dose is excreted unchanged in the urine within 6 hrs after administration, 90% is excreted within 24 hrs and 95% within 30 hrs (Pratt et al., 1975). Because of the small molecular weight and loose binding to albumin, MTX is filtered by the kidney with the urinary output of MTX proportionate to the glomerular filtration rate (Williams and Huang, 1982).

At very low plasma concentrations, MTX appears to be reabsorbed by the kidney (Huffman et al., 1973). At higher concentrations, the renal clearance of MTX is relatively constant (Huffman et al., 1973), which indicates that the antifolate is not only filtered but is also actively secreted by renal tubular cells (Isacoff et al., 1976). The tubular secretion of MTX is inhibited by the administration of leucovorin at a dose of 20 mg/kg⁻¹ (Bertino et al., 1971).

The renal toxicity of MTX at 1 g dose is of very rare occurrence (Bourke et al., 1975). 7-OH-MTX, a metabolite of MTX is 3-5 fold less soluble than MTX, and its formation is an important factor in renal toxicity (Allegra, 1990).
One to 2% of an intravenously administered dose is excreted in the stool (Bleyer, 1978).

Jacobs et al. (1976) have shown in monkeys that, as a consequence of its limited solubility at acid pH, MTX may precipitate in acid urine causing renal tubular obstruction and delayed drug excretion.

MTX molecule more tightly binds to renal resorption system or protein which enables this toxic antifolate to be retained in the kidney (Deutsch and Kolhouse, 1989). Apart from this, MTX causes urinary folate wasting in humans and rats (Kremer et al., 1986).

**Antidotes to MTX**

Leucovorin (LCN) (Synonym: Folinic acid; N-5-formyl tetrahydrofolate; Calcium leucovorin; Citrovorum factor) is an useful antidote to MTX and is commonly used in cancer therapy (Rosen et al., 1974). Long term MTX therapy has a drastic effect on normal proliferating tissues also, particularly on bone marrow (causing leucopenia, anaemia and thrombocytopenia) and intestinal mucosa, and this limits the efficacy of MTX as an antitumour agent (Nahas et al., 1972). These toxic side effects are largely limited and prevented totally by the use of "rescue agents", which bypasses the blockade instituted by MTX on DHFR (Fig.iv) (Bertino et al., 1971; Black and Livingston, 1990). Intracellular pools of reduced folates affected by MTX-mediated inhibition of DHFR is overcome and replenished by LCN (Harrap and Reinshaw, 1978). The cytotoxic effect of MTX is irreversible after 40 to 48 hrs without adequate rescue (Black and Livingston, 1990). LCN ameliorates MTX-induced toxicity.
Fig. iv: Site of action of methotrexate and leucovorin

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\text{FH}_2, \text{dihydrofolate; FH}_4, \text{tetrahydrofolate; N}^{5,10}\text{CH}_2\text{FH}_4, \text{methylene tetrahydrofolate; N}^{10}\text{CHOCH}_4, \text{formyl tetrahydrofolate; dTMP, thymidylate; dUMP, deoxy uridylate; MTX (glu), MTX polyglutamate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; AICAR, 5-aminoimidazole 4-carboxamide ribonucleotide transferase; GAR, glycinamide ribonucleotide transformilase.}
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both by repleting intracellular pools of tetrahydrofolate and by competition with MTX for entry into the cell (Tishler et al., 1988). Administration of MTX with the reversing agent is called "Protection", whereas, delayed use of the latter has been referred to as "MTX rescue". LCN enters the cell by carrier-mediated transport system and there exists a competition with MTX for the same carrier components (Goldman, 1971). Goldman (1975) reported that high blood levels of LCN were required to accelerate the net efflux of the MTX from the cell and subsequently to reduce its re-entry.

LCN given after MTX administration induced synchronous recovery of DNA synthesis and this occurred when the drug level was well above DHFR content in these cells (Sirotnak et al., 1976). Plasma levels of MTX continue to fall after LCN administration, as influx is curtailed and the net rate of drug loss from tissue is greater, the net rate of exchangeable drug loss from tissue will reflect the magnitude of drug influx versus efflux (Nahas et al., 1972). Less LCN would be required to induce egress as plasma levels of MTX decrease with time (Sirotnak et al., 1976). The resumption of DNA synthesis did not occur until exchangeable levels of MTX following egress were essentially nil (Sirotnak et al., 1976).

Theoretically, LCN in low concentrations may selectively rescue normal cells which retain an active transport mechanism for reduced folates. In experimental studies, the concentration of LCN required to prevent toxicity increases in direct proportion to the concentration of MTX (Pinedo et al., 1976). LCN, further alleviates severe toxicities such as mucositis, pancytopenia and gastrointestinal hepatic dysfunctions (Harrap and Reinshaw, 1978).
**Enterotoxicity**

The early clinical use of MTX was associated with a significant incidence of severe and often fatal intestinal toxicity (Pinkerton and Milla, 1984). The high dose MTX (30 mg Kg\(^{-1}\)) caused villus atrophy and generalized malabsorption. This could be due either to a direct toxic effect on mature enterocytes or an indirect effect secondary to mitotic inhibition in crypt cells (Baird and Dossetor, 1981). It is of note that glucose absorption is particularly affected after repeated low dose treatment (Pinkerton and Milla, 1984). MTX metabolites and drug recycling may also contribute to prolonged enterotoxicity (Taminiau et al., 1980). Repeated low dose MTX therapy can cause cirrhosis and fatty liver while, high dose of MTX can lead to transaminitis (Woolley, 1983).

**Pulmonary toxicity**

Pulmonary toxicity, both acute and chronic, has been reported with MTX therapy (Acute leukemia group B study, 1969). Clarysse *et al.* (1969) described a syndrome of fever, cough, dyspnea, hypoxymia, eosinophilia, alveolar and interstitial diffuse pulmonary infiltrates with granuloma in leukemia patients receiving MTX. Mice on low chronic doses of MTX did not develop hepatic or pulmonary lesions compared to those described in humans (Freeman-Narrod and Narrod, 1977).
Reproductive toxicity

The observation that gonadal damage may occur in patients treated with cytotoxic drugs was first made by Louis et al. (1956). Defective oogenesis and spermatogenesis were encountered following MTX treatment (Koehler et al., 1986b; Bentivoglio et al, 1993; Crenin and Darney, 1993; Johnson et al., 1994).

MTX is also an abortifacient and when injected directly into the amniotic fluid and placenta (25 mg at each site) caused abortion of an 8 week intrauterine pregnancy (Buckshee and Dhond, 1992). In ectopic pregnancy, MTX therapy has effects on corpus luteum activity. A continuous decrease in the levels of β-hCG, 17β-estradiol and progesterone in plasma was observed (Shulman et al., 1992). In premenopausal breast cancer patients, MTX with cyclophosphamide and 5-fluorouracil therapy for nine long months resulted in ovarian failure, amenorrhea, decrease in the serum concentrations of estrone, 17β-estradiol, prolactin, elevation in FSH and LH plasma levels and no change in serum testosterone level (Bianco, 1985). However, Bhatavadekar et al. (1992) reported no change in pituitary and adrenal functions in these patients.

Sussman and Leonard (1980) reported the incidence of reversible oligospermia in psoriatic patients undergoing MTX therapy. These authors have also reported that MTX-related testicular injury is confined to the germinal epithelium without any alterations in Leydig cell function and secretion of testosterone. During MTX administration, LH and testosterone levels in the blood were normal, indicating no apparent effect on steroid production in testis (Sussman and Leonard, 1980). These authors have
suggested that MTX seems to interfere with spermatogenesis as a consequence of inhibition of DNA synthesis and its antimitotic action.

Van Scott and Reinerston (1959) have reported a depression in the sperm count in patients treated continuously for two weeks of an intravenous injection of MTX. De Luca et al. (1971) reported minimal or no suppression of spermatogenesis during MTX treatment. Grunnet et al. (1977) proposed that oligospermia seen in few patients receiving MTX therapy for psoriasis was not related to drug administration and it may be due to the severity of psoriasis. On the contrary, Gunther (1970) reported no significant change in sperm concentration, motility or morphology in human semen samples during long term MTX therapy.

Fox and Fox (1967) opined that majority of stages in spermatogenic cycle was suppressed by a general decrease in spermatogonial kinetic rate. MTX treated mice testis revealed a moderate tubular atrophy and decreased spermatogenesis. Litters were few when they were undergoing treatment, and withdrawal showed an increase in the litter size (Freeman-Narrod and Narrod, 1977).

Aminopterin (parent compound of MTX) treatment to rats produced marked degenerative changes and inhibition of spermatogenesis. Nucleoprotein synthesis was inhibited and as a result, frequency of metaphase in meiosis I and meiosis II was reduced. Meiotic divisions were more susceptible to aminopterin than was mitosis (Mathur et al., 1977). Besides causing arrest of cell division, folic acid deficiency resulted in the production of chromosomal abnormalities. The most common was the presence of sticky chromosomes in
metaphase. This stickiness arose from denaturation and depolymerization of DNA on the surface of the chromosome (Mathur et al., 1977).

MTX administration caused greater testicular lesions than cyclophosphamide in rats; the damage was particularly evident in tubular membranes with intense edema, hyalinosis and disseminated ruptures; it was also evident in the cell structure with "plate core", and cytoplasmic basophilia (Bentivoglio et al., 1993).

Experimental studies by Koehler et al. (1986a,b) reported a germinal cell line degeneration, modification in seminiferous tubular morphology and fertility after repeated MTX administration in rabbits. In the former study hormonal profiles revealed elevated plasma FSH level after single (57.5 mg/kg body weight) and repeated (6 mg/kg body weight, once a week for 14 weeks) doses of MTX. These authors have suggested an enzymatic defect in the gonadal steroid synthesis for the elevated plasma androstenedione, reduced plasma testosterone but unchanged serum LH level in rabbits that received repeated doses of MTX treatment.

MTX was also shown to be ineffective on 5α-reductase activity, an enzyme involved in the conversion of testosterone to dihydrotestosterone and androstenediol (Takeda et al., 1985).

Anticancer drugs are well known to produce a number of acute but reversible toxic effects on normal tissues. Most commonly affected are those organs containing self-renewing cell populations such as bone marrow, skin and gastrointestinal tract. Generally, there is no persistent or recurrent
dysfunction of these organs, once chemotherapy has been completed. There has been far less recognition of the impact of chemotherapy on other organ systems, particularly, the gonads (Morris and Shalet, 1990). The lack of attention has stemmed partly from the absence of any acute or life-threatening symptoms accompanying changes in testicular function, and in part, it has been related to the absence, until recently, of a group of long-term survivors concerned about their reproductive potential after the completion of chemotherapy for neoplastic diseases. However, in patients receiving MTX therapy for non-neoplastic diseases, reproductive failure is an unavoidable and unexpected side effect. As this drug has been widely used clinically, MTX-induced reproductive toxicity is currently being identified as a new thrust area and provided a great impetus to study the effects of MTX on primary male sex organ, the testis.

1.2. Literature pertinent to testis

Testis is the primary male sex organ which encompasses two major compartments viz. the interstitium and the seminiferous tubules (Pilsworth and Setchell, 1981; Russell et al., 1990). The interstitium contains Leydig cells (von Leydig, 1850), fibroblasts, macrophages, blood vessels, capillaries and lymphatic space bounded by endothelial cells (Fawcett, 1973; de Kretser and Kerr, 1988). The seminiferous tubules are avascular and contain different types of germ cells embedded in the lateral and apical cytoplasmic processes of Sertoli cells (Parvinen, 1982; de Kretser and Kerr, 1988). In between the two compartments and surrounding the seminiferous tubules, peritubular myoid cells and acellular substances like collagen,
fibronectin and laminin form the boundary tissue or the limiting membrane of the tubule (Clermont, 1958; Dym, 1994).

**Leydig cells** are the principal source of testicular androgens, mainly testosterone (Purvis and Hansson, 1978; Hodgson and Hudson, 1983). The interstitial tissue, as such comprises about 6% of the total volume of the testis (Christensen and Mason, 1965), and Leydig cells occupy merely 2% of the testicular volume (Mori and Christensen, 1980). Leydig cells are round or polygonal in shape consisting of smooth endoplasmic reticulum and mitochondria with tubular cristae, both of which contain the enzymes associated with steroidogenesis (Christensen, 1975). There is good correlation between the synthesis of testosterone with the amount of smooth endoplasmic reticulum in Leydig cells (Zirkin et al., 1980). Leydig cell cytoplasm also contains lipid droplets, Reinke crystals, microtubules, primary lysosomes, digestive vacuoles (secondary lysosomes) and residual bodies (late secondary lysosomes) (de Duve and Wattiaux, 1966). Each cell contains a large, round or oval nucleus with a thin rim of heterochromatin, broken only at the site of pores through the nuclear envelope. The nucleus exhibits one or two prominent nucleoli (Christensen, 1975; Hodgson and Hudson, 1983).

The growth and development pattern of Leydig cells in rats exhibit at least two distinct phases or waves, one occurring in the fetus and another at the time of male puberty (Lording and de Kretser, 1972). This biphasic development of Leydig cells coincide with changes in plasma testosterone levels which are comparatively high in fetus (Faiman et al., 1981), very low during
infancy and significantly increased during maturation (Levasseur and Thibault 1980; Faiman et al., 1981; Chase et al., 1982).

There are two populations of Leydig cells, designated as type I and type II in rat testis (Payne et al., 1980; O'Shaughnessy et al., 1981). During early sexual maturation, the increase in Leydig cells are contributed by type I and type II. Leydig cells type I decline in the later maturation period but type II continues to increase for several more days and remains constant thereafter (Chase et al., 1982). Differences in steroidogenic capacity among two types of Leydig cells could be explained considering the differences in the activities of cytochrome P$_{450}$ enzymes associated with the smooth endoplasmic reticulum (O'Shaugnessy and Payne, 1982).

Steroidogenesis in Leydig cells requires uptake or de novo synthesis of cholesterol and involves the action of 4 enzymes. The first enzyme in this pathway, the cholesterol side-chain cleavage (P$_{450}$scc), is found in the inner mitochondrial membrane and catalyses the cleavage of the side-chain of cholesterol to yield the C$_{21}$ steroid, pregnenolone. Pregnenolone diffuses across the mitochondrial membrane and is further metabolized by enzymes associated with the smooth endoplasmic reticulum. Pregnenolone is first converted to progesterone by the action of 3β-hydroxy steroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD). The next reaction catalysed by the cytochrome P$_{450}$ enzyme, P$_{450}$-17α-hydroxylase involves 17α-hydroxylation of progesterone, followed by cleavage of the C17-20 bond. This step reduces the number of carbon atoms from 21 to 19, yielding androstenedione, the immediate precursor of testosterone (Payne, 1990). The
final step is the reduction of the 17-ketone by 17-ketosteroid reductase (17β-hydroxy steroid dehydrogenase (17β-HSD)) (Bogovich and Payne, 1980).

Leydig cell contains surface receptors for LH and responds to stimulation by producing and releasing testosterone (de Kretser et al., 1971). Testosterone either diffuses into the tubule or is bound to a carrier, such as albumin, that delivers it across the lymphatic space into the seminiferous tubule (Setchell et al., 1981). LH stimulates cholesterol transport (Rommerts and Brinkman, 1981), formation of pregnenolone and testosterone in an isolated Leydig cell mitochondria (de Kretser and Kerr, 1988). Apart from LH, FSH and prolactin have important complementary roles in regulating the differentiation and function of these cells (Hansson et al., 1976). The effect of FSH on Leydig cell function is indirect (Kerr and Sharpe, 1985; Das, 1992) and probably mediated by Sertoli cell (Saez et al., 1989), the only cell which contains FSH receptor (Teerds et al., 1989; O'Shaughnessey et al., 1992). FSH has been demonstrated to increase Leydig cell LH receptors (Chen et al., 1977), stimulate certain testicular steroidogenic enzymes (Murono and Payne, 1979; Shaw et al., 1979) and augment testosterone secretion initiated by LH in immature hypophysectomized rats (Lostroh, 1969; Chen et al., 1977; Vihko et al., 1991). Aragona et al. (1977) reported the localization of prolactin receptors on Leydig cell membranes. Prolactin acts on the Leydig cells to increase its responsiveness to LH stimulation (Bex et al., 1978).

In general, testosterone is known to cause a dose-dependent inhibition of LH and FSH secretion through interaction with the hypothalamic-releasing
hormone, gonadotropin-releasing hormone (GnRH) at the level of hypothalamus (Plant et al., 1978; Sherins and Loriaux, 1973).

**Peritubular myoid cells** form a layer of flattened cells between the interstitium and the seminiferous tubules (Ross, 1967; Dym, 1994). They are in close contact with Leydig cells in the interstitium (Skinner, 1991). With Sertoli cells, the peritubular myoid cells produce an extracellular matrix (Hadley et al., 1985) and provide structural support for the spermatogenic epithelium (Skinner et al., 1985). Furthermore, these cells are involved in the contraction of the tubules (Clermont, 1958), which play a role in the release and transport of the testicular spermatozoa (Kormano and Hovatta, 1972; Anthony and Skinner, 1989). Peritubular cells, under androgenic control produce a paracrine factor, termed P-Mod-S, that modulates Sertoli cell differentiated functions (Norton and Skinner, 1989).

**Sertoli cells** are the somatic component of the seminiferous epithelium, columnar in shape extending from the basement membrane of the seminiferous tubules to its lumen (Schulze, 1974; Fawcett, 1975; Bardin et al., 1988). These cells proliferate until 16-18 days after birth, when they form the blood-testis barrier (Steinberger and Steinberger, 1971; Orth, 1982). After 16-18 days of age, the Sertoli cells develop morphologically until at 33 days after birth, their ultrastructure is similar to that seen in adults (Chemes et al., 1979).

Tight junctions at the basal area of seminiferous tubule between myoid epithelial cells form the **first blood-testis barrier for macromolecules** (Tung and Fritz, 1980). Tight junctions between adjacent Sertoli cells (Sertoli-Sertoli cell junction) act as the **second blood-testis barrier for**
micromolecules (Setchell and Waites, 1975; Russell and Peterson, 1985). One of the functions of the Sertoli cells is to create stable intratubular environment for germ cell differentiation by providing this blood-testis barrier (Dym and Fawcett, 1970; Setchell, 1980).

Sertoli cells form two permanent viz. basal and adluminal compartments and one transient (intermediate) compartment within the seminiferous epithelium (Dym and Fawcett, 1970; Steinberger and Steinberger, 1977). While basal compartment cells include spermatogonia and spermatocytes up to the early leptotene phase of meiosis, adluminal compartment consists of inner two thirds of tubules and has pachytene spermatocytes to spermatids (Russell, 1978). These cells apparently have free access to substances diffusing from the lymphatic system since the lymph surrounds the seminiferous tubule (Dym and Fawcett, 1970; Setchell, 1980). Indirectly, through lymph, basal compartment cells have access to the products delivered from the vascular system. A third compartment known as the intermediate compartment is formed during the transit of leptotene cells from the basal to the adluminal compartment and involves successive formation and breakdown of tight junctions (Russell, 1977; 1978).

The junctional contact between adjacent Sertoli cells demarcate the basal from adluminal compartment, restrict the passage of many growth factors, nutrients and hormones from the basal to the adluminal compartment of the seminiferous tubules (Bardin et al., 1988). In the adult testis, another complex interaction, referred to as desmosome-like junction forms between Sertoli cells and germ cells (Brokelmann, 1963; McGinley et al., 1979; Russell
et al., 1983b). This junction appears to form primarily between Sertoli cells and pachytene spermatocytes and are less frequent with spermatids (Russell et al., 1983b). These junctional complexes are permeable to agents with molecular weight less than 600 to 700 (Pelletier and Friend, 1983) and appear to transfer metabolic substances, such as choline between Sertoli cells and germ cells (Ziparo et al., 1982).

Ultrastructurally, Sertoli cells have an irregular outline with cytoplasmic projections surrounding adjacent germ cells (Nicander, 1967). The cellular organelles are concentrated in the basal perinuclear area. The nucleus is irregularly shaped with a prominent nucleolus (Fawcett, 1975; Chemes et al., 1979; Bardin et al., 1988). An abundance of endoplasmic reticulum, dense vacuoles and inclusion bodies are located in the cytoplasm of Sertoli cells (Ritzen et al., 1989). Other structural features of Sertoli cells include autophagic and heterophagic vacuoles and numerous primary lysosomes, which are believed to participate in phagocytosis and digestion of degenerating germ cells (Bardin et al., 1988).

Sertoli cell provides much of the framework for the organization of the seminiferous epithelium (Orth and Christensen, 1977; Jutte et al., 1982). The columnar and convoluted structure of the Sertoli cells, which extends from the basal to apical surface of the seminiferous tubule provide the physical support for the spermatogonia undergoing mitosis, the spermatocytes undergoing meiosis and the spermatids undergoing the process of spermiogenesis to become spermatozoa (Bellve, 1979; Russell et al., 1987). The Sertoli cell is also required to maintain the germ cell syncytium that connects all the cells
derived from an initial clone of cells (Skinner, 1991). The Sertoli cell-germ cell gap junction is thought to allow cell-cell communication (Sharpe, 1983; Skinner, 1991). Sertoli cells also have the enzyme machinery necessary for steroidogenesis, particularly aromatase, which converts testosterone into estrogen (Hall et al., 1969). Sertoli cells have been described as the 'nurse cells' of testis and their primary function has been shown to provide essential factors and creating the proper micro-environment for the development of germ cells (Fritz, 1978; Papadopoulos and Dym, 1994).

The structural complexity observed in the nuclear chromatin and Golgi complex suggest that Sertoli cells may secrete up to hundred proteins (Bardin et al., 1988). These include inhibin, which plays a major role in feedback to the pituitary to selectively inhibit the secretion of FSH (Rivier et al., 1991), activin - stimulator of FSH from pituitary, androgen binding protein (ABP), carrier of testosterone and dihydrotestosterone (Welsh and Wiebe, 1978; Joseph, 1994), transferrin (Skinner et al., 1984), plasminogen activator (Lacroix and Fritz, 1982), myo-inositol (Robinson and Fritz, 1979), Insulin-like growth factor-I (Smith et al., 1987), sulfated glycoproteins (Collard and Griswold, 1987) and proteinaceous factors that stimulate Leydig cell steroidogenesis and DNA synthesis. Lactate and pyruvate are also produced by the Sertoli cells from substrates delivered to it from the vascular system (Mita and Hall, 1982). Lactate would then be delivered directly to the germ cells, which is their preferred energy substrate (Jutte et al., 1982).

FSH is the major regulator of Sertoli cell structure and function (Means, 1975). Specific binding sites for FSH have been reported on Sertoli cells (de
Kretser and Kerr, 1988). FSH has been shown to stimulate the production of androgen-binding protein (ABP) by Sertoli cells (Purvis and Hansson, 1981). The stimulatory effect of FSH on ABP production is enhanced by LH (Joseph, 1994). However, Hansson et al. (1974) reported that the stimulation of ABP both in intact and hypophysectomized animals is specific for FSH as other pituitary hormones or sex steroids could not increase ABP. Receptors for testosterone are present only in the Sertoli cells (Sanborn et al., 1977; Tindall et al., 1977).

Spermatogenesis is a synchronized process by which undifferentiated germ cells are transformed into spermatozoa (Roosen-Runge and Giesel, 1950; Steinberger, 1971; Kierszenbaum, 1994). The cycle of spermatogenesis begins with mitotic division of spermatogonia, proceeds through meiosis and finally ends with the release of sperms (Clermont, 1972; Russell et al., 1990). The rat seminiferous epithelium has 14 morphologically distinct stages, based on the morphological changes in the spermatid (Leblond and Clermont, 1952; Perey et al., 1961). The time required for one sequence through all of the stages constitutes a spermatogenic cycle (Clermont, 1963). In Wistar strain rats, it was observed to be 53.2 days (Huckins, 1965).

Spermatogonia have been classified into three types: A type, intermediate and B type spermatogonia (Huckins, 1971). Ap spermatogonia are considered as renewing stem cells, providing the spermatogonial pool for spermatogenesis (Clermont, 1972). The pachytene spermatocytes represent the longest phase of the prophase of the first division of meiosis and may be observed during the first twelve stages of the
cycle of the seminiferous epithelium (Leblond and Clermont, 1952). At stage XIII, the pachytene spermatocytes become diplotene spermatocytes and quickly terminate anaphase I and telophase I giving rise to two secondary spermatocytes. The latter exists for only a few hours and they undergo the second division of meiosis, giving rise to four round spermatids (Perey et al., 1961).

FSH and testosterone have been implicated as regulators of mammalian spermatogenesis (Clermont and Harvey, 1967; Russell et al., 1987). FSH plays a role in the development of spermatogonia and early spermatocytes in immature rats as seen by a decrease in the number of these cell types in rats treated with anti-FSH antibodies (Chemes et al., 1979). However, rats treated with anti-FSH showed no disruption in the process of meiosis and it is concluded that meiosis can proceed independently of FSH (Chemes et al., 1979). It has also been suggested that FSH may play a role in spermiogenesis (Steinberger, 1971; Bartlett et al., 1989).

The importance of FSH in the initiation of spermatogenesis during pubertal development is well-recognized (Raj and Dym, 1976; Russell et al., 1987). During this phase of development, testosterone and FSH appear to have a synergistic effect, rather than each acting independently at different phases in the development of germinal cells (Russell et al., 1987). FSH may be a prerequisite for testosterone action during puberty.

The principal action of testosterone is to facilitate the maturation of round to elongated spermatids during spermiogenesis (O'Donnell et al., 1994). Testosterone also acts to stimulate the spermatid binding to Sertoli cells
specifically at the transition from stages VII to VIII of the seminiferous epithelium (Cameron et al., 1993). Recent studies have shown that spermatogenic cells including round spermatids internalise testosterone bound to androgen-binding protein via receptor-mediated endocytosis and this process is maximal at stages VII and VIII (Gerard et al., 1994; O'Donnell et al., 1994).

The dependence of the normal spermatogenic process on continued stimulation of the pituitary gonadotropins has been well established, since following hypophysectomy, the testes of rats of different ages rapidly regressed and spermatogenesis was halted (Clermont and Harvey, 1965). The administration of gonadotropins or testosterone to hypophysectomised animals, however prevented to variable degrees the massive regression of the seminiferous tubules (Boccabella, 1963; Steinberger and Duckett, 1967). FSH replacement to immature hypophysectomised rats not only stimulates seminiferous tubule growth, but also induces Leydig cell hypertrophy and hyperplasia and it increases testicular LH receptor number and the steroidogenic response of Leydig cells to LH (Kerr and Sharpe, 1985; Chen et al., 1977).

Following hypophysectomy, as a consequence of lowered LH, testosterone production by Leydig cells decreases progressively and the testis becomes smaller (van Beurden et al., 1976). It has been suggested that estradiol is produced by Sertoli cells and that this hormone regulates FSH production and/or release (Walsh et al., 1973). In addition to hormones, cell-to-cell interactions within the testis among germ cells, Sertoli cells, peritubular myoid
cells, and Leydig cells are considered to participate in the regulation of spermatogenesis (Skinner, 1991).

Several biochemical processes are known to be specific to certain stages of spermatogenesis and to certain cell types. DNA synthesis is essential for cell division and functional differentiation in testicular germ cells (Söderström and Parvinen, 1976a; Clausen et al., 1982).

In rats, middle and late pachytene spermatocytes are the most synthetic of all germ cell types with RNA synthesis peaking at mid-pachytene and remaining high thereafter until late pachytene (Söderström and Parvinen, 1976b). The intense activity of RNA in the cytoplasm of spermatogonia and primary spermatocytes confirm that these two stages of spermatogenesis are mostly concerned with protein synthesis (Söderström and Parvinen, 1976b).

Monesi (1964) showed that RNA synthesis varies markedly in the course of mouse spermatogenesis. A high rate of RNA synthesis was observed in type A spermatogonium, while it gradually decreased in intermediate and type B spermatogonia and in preleptotene spermatocytes. No RNA synthesis was seen in leptotene, zygotene or early pachytene spermatocytes, while a sudden increase occurred at the mid pachytene stage. The RNA synthetic rate gradually decreases towards the end of the pachytene stage, and cells in diplotene and meiotic reduction divisions do not synthesise RNA at all. A low rate of RNA synthesis was detected in late spermiogenesis after condensation of the chromatin.
RNA synthesised in the seminiferous tubules was found to be mostly heterogenous nuclear RNA (HnRNA) which has a long life time (Söderström and Parvinnen, 1976b). This includes the precursors of long lived mRNA species needed for the direction of the protein synthesis during late spermiogenesis, when no nuclear RNA synthesis occurs (Monesi, 1964). The most likely sources of the stable HnRNA in the rat seminiferous epithelium seems to be the pachytene spermatocytes, especially in stages VI-VIII, where HnRNA and also the rRNA are most actively formed. Other sources are the young spermatids, spermatogonia or peritubular myoid cells, but either the RNA synthesis in these cells or their number is too small to influence markedly the total RNA synthetic pattern (Söderström and Parvinen, 1976b).

The steroidogenic and spermatogenic functions of the testis depend on metabolic energy production by the testis (Johnson, 1970). Lipids and carbohydrates are the two major energy sources in the testis (Free, 1970).

Testis gets the pentose sugars for nucleotides and nucleic acid synthesis and reducing equivalents for lipogenesis is from the HMP shunt (Johnson, 1970). Glucose-6-phosphate dehydrogenase (G-6PDH), the first enzyme of the pentose phosphate cycle, catalyses the conversion of glucose-6-phosphate into 6-phosphogluconate. During this reaction, one molecule of NADPH is generated (Mayes, 1990). 6-phosphogluconate dehydrogenase (6-PGDH) is a cytoplasmic enzyme, catalysing the second step in the pentose-phosphate pathway, where dehydrogenation and decarboxylation of 6-phosphogluconate takes place to convert it into
D-ribulose 5-phosphate. During this reaction, a second molecule of
NADPH is generated (Mayes, 1990).

The cytoplasmic ATP-citrate lyase is thought to be involved in the
generation of acetyl CoA and oxaloacetate for lipogenesis. Malate
dehydrogenase (MDH) is involved in the conversion of oxaloacetate to
malate, during which NAD is reduced to NADH (Banks et al., 1979).
NADP-isocitrate dehydrogenase (NADP-ICDH) is vital in controlling
lipogenesis, by virtue of providing coenzyme NADPH and carbon building
blocks necessary for lipogenesis (Mayes, 1990). Hypophysectomy reduced the
activity of this enzyme in rat testis (Brown et al., 1966). In general, both ICDH
and MDH exhibit higher activity, compared to other oxidative enzymes in the
testicular cells (Free, 1970). Another important enzyme involved in NADPH
production is malic enzyme, whose activity is high in tissues with high
lipogenic activity (Pande et al., 1964). This enzyme is involved in the
formation of malate from pyruvate.

NADPH thus formed, is essential for malonyl CoA synthetic pathway in
the mitochondria and for mitochondrial and microsomal fatty acid chain
elongations (Mayes, 1990). NADPH is also required for the production of
testosterone as it is involved in the cholesterol side-chain cleavage i.e. C-27
side chain cleavage by cytochrome P_{450} (Hall, 1988) and in the reduction of
androstenedione into testosterone (Inano and Tamaoki, 1986; Hall, 1988).

It is well-known that lipids are of fundamental importance in the
development and functioning of testicular tissue (Kerr and de Kretser, 1975;
Paniagua et al., 1987). During spermatogenic arrest, the Sertoli cell becomes
prominent with accumulation of lipids, glycogen and ascorbic acid (Lynch and Scott, 1950). With reactivation of spermatogenesis, the Sertoli cell lipid inclusions disappear (Johnson, 1970; Kerr et al., 1984).

Sertoli cell contains large number of lipid droplets, generally located as tiny dust-like particles throughout the cytoplasm (Lynch and Scott, 1950). The lipid content of Sertoli cell is highly variable from species to species and is reflected in the size of lipid droplets. It is generally localized near the base of the Sertoli cells, the basal portion of the cell is crowded with lipid droplets of 2-3 µ in diameter in all stages (Paniagua et al., 1987). A number of studies have shown cyclic accumulation and depletion of Sertoli cell lipid inclusions during the rat spermatogenic cycle (Kerr and de Kretser, 1975; Marzouki and Coniglio, 1982; Kerr et al., 1984). Earlier studies showed the presence of cholesterol in the lipid droplet of Sertoli cell of rat (Perlman, 1950; Collin and Lacy, 1969).

Nearly 80% of the total lipids in the testis are composed of phospholipids and the remaining 20% of glycerides and cholesterol (Oshima and Carpenter, 1968). According to these authors, the distribution of phospholipid classes in the adult rat testis is as follows: phosphatidyl choline 47.5%; phosphatidyl ethanolamine 26.2%; phosphatidyl serine 7.2%; phosphatidyl inositol 2.3% and sphingomyelin 6%. Free cholesterol forming 95% of the total cholesterol and triglycerides were the predominant classes found in the neutral lipid fraction. Mono- and diglycerides were found in trace amounts.
In the present investigation, an attempt has been undertaken to study the effect of MTX on rat testis and its susceptibility to reproductive toxicity with special reference to its MTX effects on serum hormones, testicular histology and lipid metabolism.

1.3. Scope of the present investigation

The wide clinical use of MTX in non-neoplastic diseases provided a great impetus to evaluate its toxic effects on testis as infertility is an unavoidable and unexpected side effect in many patients (Sussman and Leonard, 1980). Having this in mind, the present study was undertaken,

(i) To determine whether the low dose MTX treatment i.e., 3 mg/kg body weight, a dose commonly employed in the non-neoplastic diseases, produce any reproductive toxicity, with particular reference to testis.

(ii) Secondly in clinical studies, weekly injections of MTX therapy are reported to be less toxic as compared to the daily injections (Pinedo, 1978). Therefore, the effects of single dose, 4 consecutive days, 4 and 8 weekly doses of MTX treatment were assessed.

(iii) Thirdly, the toxic side effects of MTX have largely been ameliorated by the use of a rescue agent, leucovorin (LCN). Therefore, in the present investigation, along with the study of the effect of MTX, an equal emphasis has been laid to evaluate the effects of LCN supplementation on testis.
The existing reports on clinical studies are conflicting, which may be attributed to the variations in age, duration of treatment and degree of etiological factors encountered in patients undergoing MTX therapy for various non-neoplastic diseases. Further, as most of the studies are confined to clinical cases, the number of cases studied are not substantial and the parameters taken into consideration are not sound to explicate the action of drug on male reproduction. Nevertheless, in both clinical and experimental studies, there is consensus that one of the major effects of MTX in relation to reproduction is modification in androgen status. Therefore, to evaluate the steroidogenic potency of the organ, studies on (i) serum hormones of hypothalamo-hypophyseal-testicular axis (FSH, LH, testosterone and estradiol), (ii) steroidogenic enzymes (3β and 17β-hydroxy steroid dehydrogenases), (iii) substrates (cholesterol and its fractions) and (iv) enzymes involved in pyruvate-malate cycle (NADP-isocitrate dehydrogenase, malic enzyme, malate dehydrogenase and ATP-citrate lyase) which are functionally related to lipogenesis and generate NADPH, (cofactor in steroidogenesis) were considered.

Histological studies were carried out to delineate the effect of MTX on spermatogenesis. Studies on various lipid classes (glycerides, phospholipids) were taken into consideration as they serve as energy source for various germ cell types (Oshima and Carpenter, 1968) and stabilize germ cell membrane structure (Yoke et al., 1969).

Studies on the above parameters are expected to throw light on the mechanism of action of the drug, behind MTX-induced reproductive toxicity.