List of abbreviations

ANOVA	Analysis of variance.
DNA	Deoxyribonucleic acid.
DNMTase	Deoxyribonucleic acid-methyltransferase.
dNTPs	Deoxynucleotidetriphosphate.
Fibroblast like AME 11D1/2 cells	Fibroblast like cells of Asal mice embryos at the age of 11.5 days old.
FCS	Fetal calf serum.
FSH	Follicle stimulating hormone.
HAT	Hypoxanthine aminopterin and thymidine medium.
HPRT	Hypoxanthine guanine phosphoribosyl transferase.
LH	Leutinizing hormone.
MTHFR	Methylene tetrahydro-folate reductase.
PCR	Polymerase chain reaction.
PBS	Phosphate buffer saline.
RAPD	Randomly amplified polymorphic DNA.

Chapter Five

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Chapter Four

Discussion

4.1- Detection of the estrous cycle in the white mice female:

Puberty is the culmination of the physiological and behavioral changes associated with the maturation of the mouse reproductive system (Whittingham and Wood, 1983). Mouse female maturation is characterized by commence of the estrous cycle and ovulation. Whereas, mouse male maturation is characterized by full spermatogenic activity and attaining their secretions from the accessory glands.

Smith and Engle (1975) had reported that, the onset of mouse female puberty occurs from about four weeks onward coincides with the rising in the levels of gonadotropins regulated by the hormonal secretions of the hypothalamus. So that, female estrous cycle was dependent upon a complex integration of pituitary, ovarian and adrenal hormones under the control of the hypothalamus (Richards, 1994). While the hormonal control of the male spermatogenesis was dependent upon the testosterone, androgen together with the luteinzing hormone (LH) (Bellve *et al.*, 1977).

Several reports indicated that, there was a considerable amount of inter-strain variability in the age of puberty in both males and females (Lam *et al.*, 1970 and Krarup *et al.*, 1969). However, in laboratory animals, mating was limited during estrous, and Hafez (1987) demonstrated that, the duration of estrous was species – dependent and varied slightly from one female to another within the same species, which was also the truth in respect to the time of ovulation.

The length of estrous and the time of ovulation also varied in relation to internal and external factors (Hafez, 1987).

One of the first signs of the onset t of mouse female puberty is the opening of the vagina which is found to occur as early as (24) days of age in C57BL / 6J (Whittingham and Wood, 1983). While, the first estrous was found to occur until sometime afterward (between 2 and 10 days) in other strains (Donovan, 1994). Relative studies showed that, puberty occurred slightly later in mouse male (up to 2 weeks old), and the presence of adult males together with the immature males retarded their sexual maturity (Bellve *et al.*, 1977).

On the other hand, other experiments showed that, the exposure of immature females to the urine of adult males hastened puberty. Whereas, their exposure to the urine of adult females retarded puberty (Peters *et al.*, 1973).

Other environmental influences implicated in the onset of puberty where, the day length, temperature, size and sex ratio of the litters from which the females and males were originating (Whittingham and Wood, 1983).

Thereupon, the experimental mice used throughout this work were selected at the age of eight weeks old and this consideration of age was relayed on the interaction of the previously mentioned external stimuli together with the interstrain variations mentioned earlier.

Thereafter, attempts were made through this research to investigate the estrous cycle of the experimental females.

The cyclic events occurring in the mouse female ovary was found to be correlated with the anatomical changes in the reproductive tract of the mature, nonpregnant females were reflected by the cellular content of the vaginal smears (Whittingham and Wood, 1983). Accordingly, the estrous of the mice females used in this work was divided into a sequence of four phases which nameably were (proestrus, estrus, metestrus and diestrus), each phase being distinguished by changes in the cell contents of the vaginal smears.

Essentially, the proestrus phase was culminating in the ovulation-constitute of the ovarian cycle follicular phase, and reports showed that, the

leutinizing hormone (LH) surge occurred during the proestrus phase when the uterus become distended with the intraluminal fluids (Goldman *et al.,* 1973).

Therefore, the proestrus phase identified in the mouse female of this study, (figure 3.1a) showed the accumulation of different types of cells (White blood cells, nucleated epithelial cells and large cornified enucleated epithelial cells).

Dean (1982) had reported that, ovulation in the mouse female was found to occur (12 hours) after the surge of (LH) when the female vagina became cornified and one – fresh - ova cell was found in the oviducts at the first day of the estrus phase. Figure (3.1b) showed a vaginal smear taken from the females of this work at their estrus phase recognized by the accumulation of large cornified enucleated epithelial cells only.

Richards (1994) had demonstrated that, in the absence of coitus, mouse females enter the metestrus phase followed by the diestrus phase where both phases were constituting the luteal event of the cycle.

An accumulation of red blood cells was noticed in the vaginal smears made at these phases (Hafez, 1987), which were listed in figures (3.1c and 3.1d) obtained from examining the females of this work. However, it was reported by Smith and Engle (1975) that, with estrogen and progesterone levels low, the FSH and LH secretions was increased and the estrous cycle was repeated.

It was possible through this work to expect that, the estrous cycle of the experimental females occur at intervals of (4-6)days.

Other studies revealed variability in the cycle length and divided the cyclic events into six phases (proestrus, early estrus, estrus, late estrus, met estrus and diestrus) (Whittingham and Wood, 1983; Hafez, 1987).

Peters and his colleges (1973) had reported that, females were receptive to the males during the behavioral estrus only, and mating was found to occur during the dark period around the ovulation time. Therefore, the

determination of the estrus cycle events described earlier was necessary for the initiation of mating colonies.

On the other hand, the establishment of mating colonies was considered first to obtain embryos used for the initiation of the fibroblast cell cultures, and second, in the designation of the breeding groups experiments.

The number of successful mating was scored after the detection of the copulatory plugs retained in the mated females vagina. On the other hand, Whittingham and Wood (1983) had reported that, these plugs could remain in the vagina for (24 hours) after the successful mating and were consisting of secretions of the prostate, seminal vesicles and secretions of the coagulatory glands of the mated male.

4.2- Initiation of the fibroblast AME11D1/2 cell culture:

Mammalian cell cultures were widely used throughout genetic studies, and they were found to play a key role by permitting the direct observation of the cells in question (Kruse and Peterson, 1973).

Doyle *et al.*, (1990) had shown that, cultures derived from embryonic tissues survived and proliferated better than those cultures derived from adults. This was due to the lower level of specialization and the higher proliferative potential of the embryonic cells. In this respect, Davis (1994) showed that, embryonic primary cell cultures could be obtained either by allowing cells to migrate out of fragments of the tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells.

Freshney (1994) presented a procedure for preparing primary cell cultures from whole disaggregated embryos and showed that, an embryo age of around 13 days was the optical age for obtaining cultures. Therefore, the explants technique used in this work for the initiation of the fibroblast cultures relayed on the use of embryos at different ages of (9, 11-11.5 and 13) days old.

A comparition was made in the morphology and growth rate was made between the cultures obtained from the disagregation of the embryos at different ages.

In this study, embryos were disaggregated by worm trypsin which was used by many preliminary studies for the disagregation of various tissues since this enzyme was well tolerated by many cells, effective for many tissues, give the most complete disagregation and the higher cell yields due to the sensitivity of the interacting glycopeptides that mediate cells in the tissue (Doyle, 1990; Davis, 1994; Freshney, 1994).

Earlier studies showed that, it was essential for most normal primary cell cultures to be attached to a flat surface in order to grow and proliferate with maximum efficiency (Sharp, 1977; Federb and Tolbert, 1983).

Therefore, disaggregated cells were cultivated in (25 cm^3) tissue culture flasks supplemented with the cultivation medium.

Although cell cultures were obtained from embryos dissected at different ages, they were subjected to the same cultivation conditions of medium, incubation time and temperature.

Result obtained in this study showed that, the disagregation of embryos at an age of (9-10) days old produced mixed primary cell cultures. Although the ninth day old embryos contained the largest proportion of mesenchyme from which most of the fibroblastic cell cultures were derived; but most individual organs begin to form at the ninth day of gestation and they were difficult to be isolated until the eleventh day of gestation . Therefore, the large proportion of mesenchyme obtained from the disagregation of 9 days old embryos were gathered with another proportion of unwanted tissues that led to the proliferation of mixed cultures (Freshney, 1994). While, embryos dissected at (11-11.5) days old produced uniform, fibroblastic cell cultures that last viable for (6-7) passages due to the successful removal of the unwanted tissues such as (head, hands, legs and tail).

On the other hand, dissection of the unwanted organs was easier at the (13 days) old embryos; but the proportion of the mesenchyme was less than that in the previously mentioned embryos (Freshney, 1994).

Therefore, cell cultures obtained at this age were fibroblastic in morphology; but they did not last more than (5-7) days in culture.

Accordingly, it was possible to consider the (11 days) old embryos as the optimum age for obtaining successful fibroblast cell cultures.

Sharp (1977) demonstrated that, the terms "fibroblastic" and "epithelial" were loosely used in tissue culture to describe the appearance rather than the origin of cell. Thus, a bipolar or multipolar angular or spindle shaped cells, the length of which was usually twice or more its width was called (fibroblastic).

However, the observation of cultures morphology was the simplest and most direct technique used to identify cells by many workers (Feder and Tolbert, 1983; Doyle, 1990; Masters, 2000), with certain recognized short coming related to the plasticity of the cells morphology in response to different cultural conditions (Gospodarowicz *et al.*, 1978). It was found by Coon and Cohn (1966) that, alterations in the substrate and the incubation time could affect the cellular morphology.

In this study, cell cultures examined after five days of incubation gave the appearance of cells growing at patches.

While, cell cultures examined after seven days of incubation showed uniform monolayer appearance (Figures 3.2 and 3.3).

However, since the identity of the obtained cell cultures wasn’t further confirmed by DNA or protein profiles as it was recommended by Freshney (1994), the term fibroblast – like AME11D1/2 cell culture was used to describe the cell cultures obtained through this work.

Moreover, the determination of the growth rate of the fibroblast-like AME11D1/2 cell cultures was necessary for the designation of routine

subculture and for the quantification of the cellular response to different external stimuli on the culture growth and morphology.

Therefore, fibroblast – like AME11D1/2 cell cultures were seeded at a cell density of (10^4 cell/ml); Since Masters (2000) had showed that, the fibroblast cell cultures grew with a relatively uniform population of proliferating cells at a low cell density of (10^4 cell / ml) .

However, the fibroblast-like AME11D1/2 cell cultures had showed a characteristic growth pattern of (lag, log and stationary phases) when they were cultivated on RPMI1640 medium as follows:

1. In the lag phase, there was no increase in the number of seeded cells due to the period of adaptation reflected by the growing cells where cells replaced elements lost during trypsinization procedure and the cells were starting their attachment to the substrate and synthesis of new DNA was followed as it was noticed by other workers (Foley and Aftonomos, 1973; Folkman and Moscona, 1978). Then, a period of an exponential increase in the cell number was followed. Mirabelli *et al.,* (1985) had reported that, the length of the log phase was dependent on the cell type and the seeding density .
2. Towards the end of the log phase that contineoued for seven days (at about the eighth day of incubation), the fibroblast-like AME11D1/2 cell cultures reached their maximum counts and become confluent so that, all the available growth area was occupied and cells became in contact with each other.

This stage of growth was called the plateau or the stationary phase where cells became less motile in a phenomenon called "The contact inhibition" (Alberts *et al.,* 1989).

It has been realized by Folkman and Moscona (1978) that, the reduction of the normal cells growth after confluence was not solely due to contact with each other , but may also being involved in the depletion of nutrients and

growth factors in the medium. Thus, the term "density limitation" was also used to describe the motility cessation and reduced growth rate.

Therefore, a periodic medium change or subculturing were always carried out during the growth of the fibroblast-like AME11D1/2 cell cultures for the maintenance of the viability of the cell cultures once they were established.

4.2.1- Effect of different media on the growth of the fibroblast - like cell culture and the plasmacytoma SU99 cell culture:-

The composition and properties of the culture medium were the most crucial factors studied by many workers for achieving the successful cultivation of cells *in vitro* (Morton, 1970; Gospodarowicz *et al.*, 1978; Sharp, 1977). However, the choice of medium is still often empirical; So that, the selection of the medium depended on what was used by other workers for the same cells or on the currently used medium for different cells (Masters, 2000).Two types of cell cultures were used in this study (normal and transformed), in order to investigate the effect of different media on the seeding efficiency and cell morphology. Basically, culture medium had to fulfill the essential requirements of nutrients needed by the cell culture to grow *in vitro* (Freshney, 1994).

Three types of defined media were used varied in their complexity from – simple Eagle's minimal essential medium which contained the essential amino acids, vitamins and salts (Eagle's, 1959), to a rich – RPMI 1640 medium with a wide range of amino acids and other supplements (Morton, 1970), with a complex medium 199 (Morgan *et al.*, 1950).

Although it was recommended by many investigators to use Eagle's minimal essential medium for the growth of fibroblast cell lines (Dulbecco and Elkington, 1978; McKeehan *et al.*, 1981; Robert *et al.*, 1982).

It was found through this work that, the fibroblast -like cell cultures showed a demanding manner of growth reflected by the calculated seeding efficiency. Therefore, cell cultures scored a higher seeding efficiency when they were grown on the rich – RPMI 1640 medium.

Thus, it was possible to suggest that, the initiated fibroblast – like cell culture required enrichment conditions to grow which was possibly met by PRIM 1640 medium judged by the calculated seeding efficiency.

Similar observations were recorded by Gospodarowicz and his colleagues (1978) that alterations in the substrate constituents of the culture medium could affect cell growth.

However, fibroblast – like cell cultures cultivated on Eagle's minimal essential medium and RPMI 1640 medium showed typical fibroblastic morphology.

Moreover the difference in the seeding efficiencies between the two culture media was probably due to the following reasons:-

1. The glucose concentration was higher in RPMI 1640 medium (2g/L) compared to (0.1 mg / L) in Eagle's minimal essential.
2. RPMI 1640 medium contained (13) essential amino acids with additional (20) non essential amino acids. Whereas, Eagle's minimal essential medium contained the (13) essential amino acids only. Moreover, L-glutamine was required by most cells for energy and as a carbon source (Morton, 1970). The concentration of this amino acid was (0.3 g/L) in RPMI 1640 medium compared to (0.03 g/L) in Eagle's minimal essential medium.

3. The pH of RPMI 1640 medium was maintained at (7.2 – 7.4) by the double buffering system composed of (Sodium bicarbonate NaHCO3) and N-2-hydroxethyl piperazine – N -2- ethanesulphonic acid (HEPES), whereas, the pH of Eagle's minimal essential medium was maintained near neutrality by sodium bicarbonate only.

The advantage of this double buffering system was that, under low CO2 concentration, the pH of medium was maintained by an interaction between $NaHCO_3$ and HEPES. While, at high CO_2 concentrations, HEPES works alone to maintain the pH (Masters, 2000).

On the other hand, transformed plasmacytoma SU99 cell cultures showed almost the same seeding efficiency values when they were cultivated on both, RPMI 1640 medium and Eagle's minimal essential medium in addition to the typical plasmacytoma morphology.

However, the results reflected by the growth of plasmacytoma SU99 cell cultures on both media showed that, the growth rate of these malignant cells wasn't affected by the alterations of the substrate constitution of the cultivation media probably because plasmacytoma SU99 cells were transformed cells processing their characteristics mentioned by (Sharp, 1977 and Masters, 2000), which were low serum requirements, independence of growth factors, high plating efficiency, anchorage independence and growth on confluence monolayer with reduced density limitation of growth.

On the other hand, complex – medium 199 did not support the growth of both cell cultures (fibroblast – like and plasmacytoma SU99) probably due to the very low concentration of folic acid (10 Mg / L) and the low efficient buffering capacity that was dependent on the bivalent ions of calcium and Magnesium only.

However, medium 199 was recommended by Michler – Stuke and Bottenstein (1982) as a selective medium with fetal bovine serum (FBS) for the growth of "Endothelium" cell cultures, and it was used through this work

as a negative control in contrary to Eagle's minimal essential medium that was recommended by many workers as a selective medium for the growth of chick embryo, mouse embryo and human fibroblasts (Constaniniedes *et al.*, 1987 and Freshney, 1994).

4.2.2- In vitro effect of 5-azacytidine on the fibroblast-like and plasmacytoma SU99 cell cultures:

Animal cell culture systems had always played an important role in programs involved pre- clinical screening of vast numbers of chemicals for specific and non- specific cytotoxic agents, especially in the field of cancer chemotherapy where the potential value of such systems for cytotoxicity and viability testing for drug development was widely accepted (Freshney, 1978; Masters, 2000). However, the choice of the assay was dependent on the agent under the study, the nature of the response, and the particular target cell (Masters, 2000). Therefore, in this study cytotoxicity assays were carried out using two types of assays (short – term viability and long term survival assays).

Freshney (1978) used the short – term viability assay to measure the proportion of viable cells following the addition of a potential drug.

The results obtained in this work showed a cytotoxic effect of different concentrations of 5 – azacytidine on the viability of both cell cultures (fibroblast – like and plasmacytoma SU99) expressed as values of optical density measured by ELISA – reader. While, the short –term viability assay was convenient, quick and usually easy to perform; it has two dark backs: first, this assay revealed the number of dead cells only and second, an overestimation of viability might be expected due to alterations in membrane permeability (break down in membrane integrity or a perturbation of a particular metabolic pathway), which make cells permeable to uptake the dye other than being affected by the drug (Freshney *et al*., 1975 and Davis, 1994).

Fry and Bridges (1979) suggested that, when cells were subjected to antineoplastic drugs, they might show the toxic effects several hours or days later.

Accordingly, long – term survival assay was used to demonstrate the proliferative capacity of cells after, rather than, during the exposure to the drug measured in terms of plating efficiency.

It was noticed through this work that, the plating efficiency values of the fibroblast – like cell cultures was falling at higher concentration of 5 – azacytidine and they were more sensitive than plasmacytoma SU99 cell cultures to the cytotoxic effect exerted by 5-azacytidine which was might be due to the immortal character expressed by plasmacytoma cells in being transformed cells with modified extra-cellular matrix and altered cell membrane properties (Holliday, 1986).

Constaniniedes (1977) demonstrated the formation of functional striated muscle cells from non-muscle transformed fibroblastic precursors following exposure to 5- azacytidine.

Relatively, three new mesenchymal phenotypes were reported by Taylor and Jones (1979) were expressed by cultures of (3T3) and (C3H/10T1/2CL8) mouse fibroblast cell lines after treatment with 5-azacytidine. These phenotypes were characterized as contractile muscle cells, biochemically differentiated adipocytes, and chondrocytes capable of the biosynthesis of cartilage – specific proteins.

However, in this study, treatment of the fibroblast – like cells showed other results so that it was noticed that, although, treatment with 5- azacytidine initially inhibited the growth of cells, howbeit; a memory of the treatment was retained in the fibroblast – like cell cultures that survived the treatment. In other words, the sub- inhibitory concentration of 5-azacytidine had affected the phenotype of fibroblast – like cell cultures since cells appeared single with different sizes other than uniform monolayer.

However, the effect of 5- azacytidine on the phenotype of these cell cultures was different than those results mentioned by Constaniniedes (1977) or from the results declared by Holliday (1986) who had demonstrated that, treatment with 5-azacytidine had no effect on the phenotype of the transformed human fibroblast cell line.

On the other hand, Tamarin (1996) had demonstrated that, the development of specialized cells from multipotent progenitors which were distinct from the cells of the previous generation structurally and functionally was the result of a complicated array of events involved the activation and maintenance of new gene expression and silencing of the in appropriate genes. Thus, the new cellular phenotypes expressed were the result of interplay between the information encoded in the genome and the external stimuli affecting gene expression (Russo *et al.*, 1999 and Twyman, 2001). However, genes that define lineage – and differentiation of the cells in tissues were regulated by tissue – specific and temporally regulated factors (Freshney, 1985).The accessibility of these regulatory factors to the genes was dependent on the architecture of chromatin which was affected by two mechanisms: chromatin remolding by the acetyltransferases (Jones *et al*., 1983) and the methylation of cytosine (Maier *et al.*, 2003).

Therefore, the incorporation of 5- azacytidine to the newly synthesized DNA of the transformed (3T3 or C3H/10T1/2CL8) cell lines prevented the methylation of DNA and the maternal pattern of methylation was lost leading to the expression of multiple new phenotypes (Constaniniedes *et al.*, 1977; Jones *et al*., 1983 and Stopper *et al.*, 1992).While, the effect of the sub inhibitory concentration of 5- azacytidine was different on the treated fibroblast – like cell cultures probably due to the following reasons: -

1. Treatment of the fibroblast – like cell culture with 5- azacytidine was at their (5^{th}) passage since they were normal cells with a finite life span.

While, the (3T3 and C3H/10T1/CL8) cell lines were transformed cells with infinite life span and they were treated at their (23^{rd}) passage.

2. Since the fibroblast-like cell cultures were freshly explanted from mouse embryos, their 5-methylcytosince level was about (3.8%) according to (Wilson and Jones, 1983) where all the tissue – specific genes were heavily methylated (Razin and Cedar, 1993).

   Whereas, the (3T3 or C3H/10T1/CL8) cell lines were clones of immortal cells with a 5- methyl cytosine content of about (1.04 %) according to the same reference.

   Therefore, treatment of the transformed cell lines with 5- methyl cytosine led to extensive under – methylation of the genes compared to the fibroblast- like cell cultures where only substantial hypomethylationp of genes was achieved since they were heavily methylated (Jost and Saluz, 1993).

3. Taken together the fact demonstrated by Jones and his colleagues (1983) that, the transformed fibroblast cell lines rapidly lose quantities of 5- methyl cytosine during cell division and aging in culture. Thus, treatment with 5- azacytidine at the (23^{rd}) passage made many genes undermethylated which led to the expression of phenotypes.

4. In addition, Yoshimazawa (1993) reported that, the developmental abilities of mouse cells in tissue culture differed from one strain to another and since the fibroblast-like cell culture was derived from the embryonic tissues of the *Swiss albino* strain; While, the C3H/10T1/CL8 cell line was derived from BALB/c strain cell line was derived from BALB/c strain (Wilson and Jones, 1983). This might be an additional reason to the different phenotypes expressed by the treated cell cultures after exposure to 5-azacytidine.

On the other hand, Holliday (1986) had demonstrated that, treatment with 5-azacytidine was found to induce the reversion at a high rate frequency of some enzyme – deficient malignant cell lines to the wild – type phenotype. Thus, treatment of plasmacytoma SU99 cell line with 5- azacytidine induced the reversion of cells from (HPRT-ve) to the wild-type phenotype (HPRT+ve), and the reversion rate was determined by cultivating the treated cells in (HAT – medium).

Relatively, Mohandas *et al.*, (1981) had reported on obtaining the same results and demonstrated the production of HAT – resistant sub clones from a mouse – human somatic cell hybride clone (37-26R-D) deficient in HPRT after the treatment with different concentrations of 5-azacytidine.

Moreover, result obtained in this work revealed that, the reversion rate of (HAT- resistant) sub clones was dependent on the concentrations of 5-azacytidine concentrations used in the treatment, where the concentration of (3 µM) gave the higher reversion rate. The same results were obtained by Convey and Zaharko (1984) who had studied the effect of dose and duration of exposure to 5-azacytidine on a clone of leukemia cells *in vitro* and they found that, the colonogenic potential of L1210 leukemia cells was increased at one concentration (2 µM). While, the survival level of the treated cells was decreased at higher concentrations.

However, the dose – dependence reversion rate might be explained by the fact that, 5-azacytidine was highly cytotoxic to the cells in the S-phase of the cell cycle (Cihak, 1974), and it exert its action on the rapidly dividing cells (Maier *et al.*, 2003).

Therefore, at higher concentrations of 5 – azacytidine, the cytotoxic effect on the treated cells was increased rising the numbers of dead cells. Therefore, the sub inhibitory concentrations of 5 – azacytidine (3 µM and 2 µM) mentioned earlier were the appropriate concentrate ions that gave the higher reversion rates.

However, the reversion of the transformed sub clones to the wild – type phenotype after exposure to 5 – azacytidine was explained by Plumb *et al.*, (2000) who had expected that, the presence of a nitrogen atom in the 5 – position ring of the azacytidine made this pyrimidine analogue refectory to the enzymatic methylation of the DNA, making this drug a very efficient inhibitor of DNA methylation so that, 5- azacytidine molecules substituted approximately (5%) of the 5- methyl cytosine content of the cultures treated with high concentrations.

In addition, since the 5- position of the azacytidine ring wasn't involved in the hydrogen binding (Cihak, 1974). Then, 5 – azacytidine would be expected to base pair with the guanine in a regular manner leading to double strand breaks in the DNA (Kelescenyi *et al.*, 2000), and chromatide fragments exchange between homologous chromatides resulting in a kind of frame shift mutations (Boyes *et al* ., 1990).

However, results obtained with plasmacytoma SU99 cell line treatment had confirmed the explanations made earlier for the results obtained after the treatment of the fibroblast – like cell culture with 5–azacytidine; Since Poirer (2002) had reported that, treatment of the malignant cell lines with 5 – azacytidine resulted in an extensive hypomethylation of the DNA which led to the transcriptional activation of the previously silenced genes, including the hypoxanthine – guanine – phosphoribosyltrancferase genes, and additionally, since the transformed cells resembled the malignant cells (except in the invasion character) (Masters, 2000). Therefore, the acq1uistion of the multiple new phenotypes by the (3H / 10 T1 / 2 CL8) sub clone was explained by the fact of their being transformed cells with immortality features (Razin and Cedar, 1993).

4.3- In vivo effects of 5-azacytidine and folic acid on the mice:

While, the *in vitro* measurements of the 5-azacytidine toxicity had resembled purely the cellular response to events such as (time of drug exposure, drug concentration, metabolism and tissue response), and since these measurements, could be interrupted by various *in vivo* reactions due to the differences existing between the *in vitro* and *in vivo* responses, (since many drugs would be metabolized by the liver and turned non- toxic) (Alonso-Alperte and Varela-Moreiras, 2000).

In other words, for the *in vitro* studies to be effective, relative *in vivo* models were suggested in order to determine whether the results of the *in vitro* experiments were merely representing a response to the cultural conditions used through this investigation or the administration of 5-azacytidine had the same significant effects *in vivo* similar to those effects observed *in vitro*.

On the other hand, ever since the early studies of Sirotank and DeGraw (1984) on the *in vivo* effects of 5-azacytidine on mice and the experiments of Carr *et al.,* (1984) on the effect of the same agent on the DNA stability, the usefulness of this agent in determining the essentiality of the physiological methyl groups in maintaining normal development in mice was recognized.

Thereupon, attempts were made through this investigation to summarize evidences supporting the generalization that, the alteration of the DNA methylation pattern resulting from the experimental administration of 5-azacytidine *in vivo* could produce the same patterns of pathophysiological alterations resulting from the deficiency of folic acid (Poirier, 2002), and the effects of both agents on mice different organs were studied.

Doses, drug exposure duration time and the route of administration of both agents of 5-azacytidine and folic acid, were considered after the experiments of (Doerksen *et al.,* 2000 and O'Neill, 1998) respectively. While, the lethal

doses (LD50) of both agents were defined as that concentration (dose) of the agent used that caused the death of half the animals in the treated group of that specific agent (Levinson, 2004).

4.3.1- In vivo effects on mice body weight:-

It was suggested by Whittingham and Wood (1983) that, testosterone acts with the growth hormones to increase the body weight, which consequently, makes mice males heavier than mice females of the same strain.

However, the results shown in table (3.6) revealed that, treatment of mice males and females with (8mg/kg body weight) of 5-azacytidine had significantly decreased ($p<0.05$) the body weights in both sexes, with the observation that, the treated mice females had lost more body weight than the treated mice males probably due to the fact that, mice females were more sensitive to the mutagenic effects brought-up by various chemotherapeutic agents than males of the same strain (Boring *et al.*, 1994).

Relatively, similar results were obtained by Doerksen *et al.,* (2000) in which they demonstrated that, treatment of rat males with different concentrations of 5-azacytidine did not significantly affect their body weights. While, treatment of females of the same strain with this agent had significantly decreased their body weights.

On the other hand, data obtained from figure (3.10) revealed that, there was a non –significant increase ($p >0.05$) in the body weights of the mice males and females following the oral administration of (0.1mg/kg body weight) of folic acid. Similar results were reported by Kim (1999) who suggested that, the non- significant increase in body weight of the treated mice with folic acid was probably due to the cause- and effect- relation ships between the intake of vitamins and the increase in the body weight.

4.3.2- In vivo effects on mice male reproductive organs:

In spite of the increase in the effectiveness of cancer chemotherapy and the prolongation of life achieved by these agents (Raman and Naryan, 1995). It

has been reported by several workers that, these agents might have certain draw-backs of causing male sterility (Arsenau *et al.,* 1974), induction of tumorigenecity and embryo lethality in several laboratory animals (Takeuchi and Tacheuchi, 1985; Matsuda and Yastomi, 1992).

Accordingly, the possible effects of 5-azacytidine and folic acid administrations on mice males reproductive organs were assessed in this study by investigating the effect on the following terms:-

4.3.2.1- Testes histology

The results shown in figure (3.11) revealed various abnormalities detected in the seminiferous tubule of mice males treated with 5-azacytidine.Similar results were obtained by Doerksen *et al.,* (2000), in which they demonstrated that, treatment of rats males with 5-azacytidine resulted in deleterious effects on testes histology.

However, it was declared by (Lam *et al.*, 1970; Pavinen *et al.,* 1978 and Ariel *et al.,* 1991) that, the seminiferous tubule were considered abnormal if they contained (vacuoles, multinucleated giant cells, degenerating tubule, sloughing of immature germ cells into the lumen). However, it was suggested by Tesarik *et al.*, (1992) that, drugs that induce alterations in androgen secretion usually produce changes in the reproductive system such alterations that could affect the organization of the germ cells in testes.

The administration of folic acid on the other hand, had no effect on the organization of the germ cells in the seminiferous tubule of the treated mice males.

4.3.2.2- Sperm function:

The effects of 5-azacytidine and folic were studied on the following:

4.3.2.2.1- Sperm motility:

Determination of the percentage of motile sperms in the semen specimen was a critical factor in the evaluation of semen quality (Ohl and Menge, 1996).The obtained results after the intraperitonial injection of 5-azacytidine,

shown in table (3.7), revealed that, there was a decrease in sperm motility of the 5-azacytidne treated group when compared with the sperm motility of the control group. Similar results were obtained by Doerksen *et al.*, 2000

However, it was well known that, the secretion of proteins into the epididymal lumen influences sperm maturation, in which a glycoprotein presents in the epididymal luminal fluid that induces the forward motility of the caput epididymal sperms (Palladino and Hinton, 1994). Therefore, the decrease of sperm motility can be due to the effect of 5-azacytidine in alterating the secretion and function of proteins in the epididymus which caused the decline in sperm motility (Gupta *et al.*, 2002), or may be due to inhibition of enzymes activity necessary for sperm metabolism(Lee and Moon, 1982).

4.3.2.2.2- Sperm concentration:

Measurements of sperm concentrations have been used in the assessment of sperm functions (Mortimer, 1994).The obtained results in table (3.7), showed a decrease in the sperm concentration of the 5-azacytidine treated group when compared with the sperm concentration of the control group.

Similar results were reported by Doerksen *et al.*, (2000), in which they demonstrated that, androgens were essential for the initiation and maintenance of spermatogenesis, while, (LH, FSH and testosterone) were required for the control of the spermatogenesis process. Therefore, changes in the synthesis and metabolism of these hormones caused by 5-azacytidine may result in the reduction of the number of sperms in the caudal epididymus (Sharma *et al.*, 2003).

4.3.2.2.3- Percentage of dead sperms:

Determination of the percentage of dead sperms is one of the important criteria in the assessment of sperm function (Ohl and Menge, 1996).

The results of table (3.7) showed that, there was a decrease in the viability of the sperms of the 5-azacytidine treated group when compared with the percentage of the control group.

Similar results were reported by Smikle and Turek, (1997) in which they demonstrated that, viable sperms repel the vital stain (eosin-nigrosin), whereas, the dead sperms absorb the dye because they had lost the structural integrity of their plasma membrane.

4.3.2.2.4- Percentage of morphologically abnormal sperms:

Morphologically normal sperms swim faster, straighter with higher tail beat frequencies than the abnormal ones (Katz *et al.*, 1982).So if the percentage of normal motile sperms is decreased, the number of sperms reaching the upper female tract might be also decreased, thereby, decreasing the chance for fertilization (Ohl and Menge, 1996). Therefore, the morphological analysis of sperms is an important criterion to study the effects of certain drugs on sperm function (Adelman, 1986).Since some drugs exert inhibitory effects on enzymes necessary for sperm metabolism leading to the decrease or loss of motility. (Lee and Moon, 1982).

The results of figure (3.12) revealed a number of morphologically abnormal sperms obtained following the treatment of mice males with 5-azacytidine.In addition, table (3.7) showed that, the a percentage of (40%) morphologically abnormal sperms were detected in the 5-azacytidine treated male group when compared with the percentage of the control group. However, similar results were reported by Majumder *et al.*, (1990) in which they had revealed that, a percentage of more than (50%) abnormal sperm in the semen was classified as teratospermia. In addition, it was considered by La Nasa and Urry, (1985) that such a percentage of (50%) morphologically abnormal sperms was associated with sub- fertility in mice males.

On the other hand, it was reported by Seely *et al.*, (1996) that, the reproductive function of mice males, (The formation of type A spermatogonia

and the conversion of the primary spermatocytes into secondary spermatocytes by meiosis I) were under the hormonal control of (LH, FSH and testosterone). While, the final maturation step of the spermatids were dependent on FSH only (Ganog, 1991). Therefore, the recognized abnormal sperm morphology after the administration of 5-azacytidine might reflect an abnormal intratesticular maturation combined with a hormonal disturbance (Acosta *et al.*, 1`988; Jost and Saluz, 1993), probably caused by the decrease in the DNA methylation content produced by the inhibitory effect pattern exhibited by 5-azacytidine due to the lowered methylation content in the mouse sperms (Razin and Cedar, 1993).

The results of the morphological examinations that were obtained on the other hand, following treatment with folic acid showed no effect of this vitamin on the sperms morphology of the treated mice males' mouse in this study. However, similar results were emphasized by Alder and Terras, (1990).

These observations however, might indicate a condition of low fertility associated with the lowered sperm motility, concentration, viability and morphological abnormalities which were recorded in this study.

4.3.3- In vivo effects of 5-azacytidine and folic acid on the mice fertility rate and the pregnancy – out come:

Drugs that induce alterations in androgen secretion usually was found to produce changes in the reproductive system, such changes might include a low percentage of sperms exhibiting normal morphology that would greatly reduced the prognosis for fertility (Kruger *et al.*, 1986 and Arab *et al.*, 1989).

On the other hand, it was assumed since the early works of Mahadevan and Tronnson, (1984) and Tash *et al.*, (1988) that, the pattern and degree of sperm motility, concentration and morphology were the probably considered as the most crucial factors in determining the fertility rates. Accordingly, a breeding group was established in this investigation consisted of (20 mice males) treated with 5-azacytidine mated with (30 untreated mice females) and

the fertilization rate was assessed by determining the numbers of pregnant females for the next two mornings. Results of figure (3.16) had revealed a decrease in the fertility rate of the mice males treated with 5-azacytidine when compared with the control group. However, these results came in consistent with the results obtained by Arnon and his colleges (2001) in which they had reported that, mouse sperms were very sensitive to the epigenetic effects brought – up by various DNA methylation inhibitors such as 5-azacytidine. These results were probably highlighted by the fact reported by Kafri *et al.*, (1990) in which they had demonstrated that, the DNA of the germ cells of both mice males and females was differentially methylated in a manner made the sperms more sensitive to the mutagenic effects than females' germ cell.results of table (3.13) and figure(3.17)had confirmed the above explanations.However, results of figure (3.16) had also showed that, treatment of mice males with folic acid had no effect on their fertility rate where the administration of this vitamin had no effects on the male reproductive system.

On the other hand, the possible relation ship between the effect of maternal exposure to 5-azacytidine and folic acid on the embryonic development was also investigated in this study, since it has been shown by Lash and Whittacker, (1974) and Bressman and Seto, (1976) that, mammalian embryonic development required sequentially ordered genetic steps which might be affected by various chemical agents.

Results shown in table (3.10) revealed that, there was an increase in the (number of resorbed embryos, pre-implantation losses and post- implantation losses) in the 5-azacytidine treated group when compared with the control group. Similar results were obtained by Doerksen and Trasler, (1995) and Paynton *et al.*, (1988), in which they had reported that, in the mouse as in many mammals, early embryonic development was dependent upon the contribution of both maternal and embryonic genomes. Therefore, it was suggested that, maternal exposure to 5-azacytidine at their (7^{th},8^{th} , 9^{th} , and

10^{th}) days of pregnancy was detrimental to the embryos assessed in terms of resorbed embryos , pre- and post- implantation losses.

On the other hand, results of table (3.11) showed that, treatment of pregnant females with folic acid alone did not affect the normal embryonic development. Whereas, when the treatment was combined with a heat treatment for (8.5 minutes), results revealed that, the supplementation of folic acid prior to heat treatment was necessary to avoid the increase in the embryo lethality recorded in the non-supplemented group.

However, similar results were obtained by Czeil and Dudas, (1992) and Pugarelli *et al.*, (1999) in which they had demonstrated that, folic acid antagonists such as (methotrexate, pyrimethamine and trimethoprime) were associated with various birth defects in both mammals and mice. In addition , recent clinical reports made by Shin and Shiota ,(1999) showed that, a substantial proportion of neural tube defects could be prevented by folic acid administration prior to conception and during the early months of pregnancy.

4.3.4- In vivo effects of 5-azacytidine and folic acid on the chromosomes of the mice bone marrow somatic cells :

Bone marrow cells were considered as one of the most proliferative, self-renewing somatic cells in the mammalian body (Marguardt and Bager, 1977), in addition, to the sensitivity reflected by these cells to chemical threats reflected by chromosomes damage of the exposed cells (Tic and Levett, 1985). Moreover, these cells were used as a good indicative for the clastogenic effects brought-up by many chemicals (Tucker and Preston, 1996).

Results shown in figure (3.18) demonstrated a significant increase ($p<0.05$) in the mitotic activity of the somatic cells prepared from mice treated with 5-azacytidine when compared with the mitotic activity of the control mice somatic cells. However, these results came in agreement with the results obtained by Haaf, (1995) in which he reported that, treatment with

5-azacytidine produced dramatic effects on the chromosomes and lymphatic cells leading to the decondensation of the chromatin structure, chromosomal instability and an advanced in the replicating time of the exposed cells.

Therefore, based on the obtained results it was suggested that, the detected change in the replicating behavior observed in the somatic cells of treated mice bone marrow with the structural abnormalities detected following 5-azacytidine administration might probably indicate that, 5-azacytidine had triggered the process of cellular programming by affecting the chromatin structure through the demethylation activity which made the chromos of the bone marrow a part of their differentiated behavioral function. Thus, in a conclusion, it was suggested that the administration of high doses of 5- azacytidine could induce the tumorigenic cell transformation.

On the other hand, results of figure (3.32) obtained after folic acid administration showed no profound effect of this vitamin on the chromosomes mitotic activity. However, similar results were obtained by Duthei *et al.,* (2002) in which they demonstrated that, the dietary supplementation of folic acid was necessary for chromosomes stability.

While, the experiments of Cort *et al.*, (2001) and Wang and Fenech, (2003) had revealed that, the deficiency of folic acid had an impact on the DNA stability principally through two potential pathways; altered DNA methylation pattern and inhibited cell proliferation.

4.3.5- In vivo effects of 5-azacytidine, folic acid and on the genomic DNA methylation pattern and the cytoplasmic protein content of mice liver:

4.3.5.1- Effects on genomic DNA methylation:

The mouse liver was considered by many workers as a good model to investigate the effects of chemotherapeutic agents on the progression of experimentally induced diseases (Kutob and Plaa, 1962).

The liver DNA, on the other hand, was found (as all the mammalian genome) to contain two types of informations: - epigenetic and genetic (Jost and Saluz, 1993).While, the epigenetic component was found to provide the temporal and spatial frame –work for the genetic informations to be used (Alexiou and Leese, 1992), the genetic portion on the other hand, comprises the blue print for the synthesis of all the necessary proteins of life (Finnell *et al.,* 2002).

4.3.5.1.1- Isolation of DNA from mice liver:

Liver DNA was isolated according to the method of Sambrooke *et al.,* (1989), which showed a number of advantages involved in the steps of DNA isolation as follows: -

1. The first step involved the addition of the extraction buffer containing the following :-
    i. Tris-Cl, in order to disintegrate the cellular membranes.
    ii. EDTA, which stops the DNA's from working and withdraws the magnesium ion that affects the PCR optimization conditions.

ii. Pancreatic RNA's, which removes the contaminating RNA.

iii. SDS, as a detergent that is associated with the removal of the contaminating proteins.

2. The second step involved the addition of protinase k in sufficient quantities to degrade proteins that were left in the sample after the extraction step.
3. Since protein contamination was found to affect the DNA purity, thus, the DNA was further purified with phenol chloroform mixture.
4. The forth step involved the addition of ethanol to precipitate the DNA.
5. The final step of DNA isolation involved the quantification of DNA concentration with spectrophotometer.

Spectrophotometric measurements were made by taking readings at two wave lengths (260 and 280 nm), according to Sambrook *et al.,* (1989).

Readings taken at theses wave lengths allowed the calculation of DNA concentration in each sample since an optical density of (1) corresponded approximately to (50μg/µl) for the double stranded DNA. Thus, the ratio between the readings (OD260/OD280), provided an estimation of the DNA purity in the sample. Results were shown in table (3.14) revealed that, the purity of the DNA samples isolated from the livers treated with 5-azacytidine was lower than the purity of the DNA samples isolated from the control mouse liver or the liver of the mouse treated with folic acid. However, this result might be explained by the fact that, the DNA was heavily contaminated with 5-azacytidine substances which probably absorbed the Ultra violet irradiation and impeded the accurate analysis of the DNA purity in these samples.

4.3.5.1.2- DNA digestion with HpaII and MspI restriction enzymes:

The level of DNA methylation in the liver of the treated samples with (5-azacytidine and folic acid) was monitored using methylation sensitive and

non-sensitive isoschizomers (HpaII and MspI) restriction enzymes according to the method mentioned by Bird and Southern, (1978) and Waalwijk and Flavel, (1978).

The size distribution of the DNA fragments on agarose gel was shown in figure (3.20), which revealed the following results:-

1. Lanes(C and F), showed a distribution of a single double stranded DNA fragments with a molecular weight of (5.077 kb), which might indicate that, the liver DNA was not affected by the treatments with folic acid, since the same size distribution of the DNA fragments were detected in the treated DNA lanes when compared with the lane of the control DNA, in other words the DNA remained in its intact form no matter of the treatments with the above agent. Whereas, lane (A), showed the size distribution of two fragments with the molecular sizes of (11.497 and 5.077 kb).

   This difference in the size distribution of DNA fragments was probably due to the ability of 5-azacytidine to induce heritable changes in the gene leading to DNA instability and breakage (Haaf, 1995).
2. Since HpaII is a methylation sensitive enzyme which cleaves the sequence 5-CCGG-3, but it does not cleave it when the internal cytosines are methylated (Singer et al., 1979). Thus, lanes (CH and FH) yielded fragments with the molecular sizes of (11.497 and 5.007 kb) .While, the isoschizomer MspI which is a methylation non- sensitive enzyme that cleaves the same DNA sequence irrespective of its methylation status (Waalwijk and Flavell, 1978), had generated fragments in lanes (CM and FM) with the molecular sizes of (11.497, 5.077, 4.479 and 4.507 kb).
3. On the other hand, lanes (AH and AM) yielded fragments much larger in size than the above mentioned fragments with no detectable difference between the cutting pattern of the two enzymes.

Moreover, smearness was observed of the large fragments in lanes (AH and AM) which was not due to the incomplete digestion that was ascertained in two ways: -

- The same concentration of DNA of (15 µg /µl) was considered in all samples that were digested with (2 units) of each enzyme.
- The same DNA fragment size distribution was observed in lanes (CH, FH, CM and FM).

Therefore, another explanation was suggested to explain the pattern similarity of cleavage observed between AH and AM.

Since one enzyme was methylation sensitive while the other was not:

The DNA that was digested with HpaII was assumed to be demethylated due to the action of 5-azacytidine on the treated mouse liver. While the DNA that was digested by MspI was also demethylated by 5-azacytidine but since this enzyme was methylation non-sensitive, thus, a similarity in the digestion pattern was found.

On the other hand, it was demonstrated by Sambrook *et al*., (1989) that, large DNA molecules of linear, double – stranded form would migrate more slowly than smaller one in gels because they need to worm their way through the pores of the gel less efficiently than smaller ones. Therefore, the migration of fragments in lanes (AH and AM) was limited by their large size which generated the smearness form that was observed. Similar results were obtained by Singer - Sam *et al*., (1990) in which they had improved the sensitivity of detecting the DNA methylation pattern change using RAPD-PCR analysis.

4.3.5.1.3- <u>RAPD –PCR analysis:</u>

An assay was carried out in this investigation based on predigesting the DNA's isolated from the treated liver samples with the (HpaII and MspI) enzymes prior to RAPD-PCR analysis.

However, RAPD-PCR technique was based upon the fact that, a randomly selected decamer (primers), when they were mixed with genomic DNA samples (template) with a thermostable DNA polymerase enzyme (Taq), if they were subjected to temperature, this will allow the amplification of the DNA to several fragments (Williams *et al.*, 1990).

In addition, this molecular marker technique had the advantage of that, during the reaction, primers will bind to the enzymatically digested DNA in two separated regions which will allow their amplification (Innis *et al.*, 1990) and the product was analyzed on agarose gel according to Devos and Gale, (1992).

Results of RAPD-PCR showed no detectable amplification products when the primers (OP-02, OP-12, OPA-13, OPB-12 andOPD-20) were used in the reaction.

Similar results were reported by Singer – Sam *et al.*, (1990) in which they had scored the failure of a number of decamer primers in the amplification of DNA fragments obtained from mouse liver samples and they had attributed the failures of these primers to amplify the DNA to the absence of suitable priming sites for these primers in the genome (there were no complementary sequences for these primers in the genome).

On the other hand, results shown in figure (3.21) revealed in addition to the DNA amplification, a polymorphism was also detected in the banding pattern analyzed on agarose gel using the primer OPE-7, probably due to beginning of the sequence of this primer with CCG-, which resembles in fact the beginning of the restriction enzymes recognition sites (CCGG) the sequence that possibly had provided a priming site for this primer that resulted in the right amplification of the DNA.

However, the results obtained using RAPD-PCR analysis had confirmed the previously obtained results in this investigation using HpaII and MspI restriction enzymes, where a similarity was recognized in the amplification

patterns between the DNA's isolated from the mice livers treated with folic acid when compared with the amplification pattern of the DNA isolated from the control mouse liver.

Whereas, a polymorphism was detected in the banding pattern of the DNA isolated from the liver treated with 5-azacytidine.

Similar results were obtained by Singer–Sam et al.,(1990) in which they had attributed the result of the banding pattern polymorphism to probably the following reasons: -

1. Missing bands might be resulted from insertion or deletions in the amplification region.
2. Base changes from insertions or deletions which might alter the primer binding site (Wigand *et al.,* 1993)

4.3.5.2- Effects on the cytoplasmic proteins:

It was reported by Bachman et al., (2002) that, the interaction of certain chemotherapeutic agents with the DNA - binding transcription factors (the crucial determinants of gene expression), could trigger a chain of events involved in the structure of chromatin and the assembly of divergent proteins. Therefore, an investigation was carried out to examine the effects of (5-azacytidine and folic acid) on the gene expression which was assessed by the possible changes in the liver cytoplasmic proteins phenotype using SDS- gel electrophoresis technique.

However, the application of the sodium dodecyl sulphate – polyacrylamid gel electrophoresis (SDS-PAGE), seemed to be an ideal technique to analyze the possible changes in the cytoplasmic proteins, since it has long been recognized that, this technique contained a powerful DNA denaturant and a solubilizing agent (SDS), which induces conformational changes in the proteins (Baylin and Herman, 2000) In addition, the strong effect exerted by SDS, makes essentially, all the proteins accessible for electrophoresis analysis including the normally insoluble ones (Westermeier *et al.,* 2001 and Jungblut

et al., 1990) on the other hand, had demonstrated that, the SDS-protein interaction was strong enough to make the composition of the SDS-protein complex, pH-independent and eliminated the charge differences between different proteins.

Moreover, several workers in the field of protein analysis had found that, at SDS concentrations higher than (8*104 M) the detergent will bind to proteins resulting in a complex with a rod-like shape, the length of which varies uniquely with the polypeptide molecular weights. (Jungblut *et al.,* 1990; Baylin and Herman, 2000 ; Bird *et al.,* 2002).

On the other hand, it was demonstrated by Shapiro *et al.,* (1967) and Weber and Osborn (1969) that, proteins that were dissolved in high concentrations of SDS, had exhibited electrophoresis mobilities in polyacrylamid gels which were a direct function of their sub-unit molecular weights .

However, the results of protein analysis obtained through this study using the SDS-PAGE, shown in figure (3.22), had revealed a remarkable similarity in the cytoplasmic banding pattern between lanes (1 and 3). Whereas, lane(2), showed quantitative increase in a set of two middle bands.

Based on the compilation of several published data (Chapman *et.al;,* 1984; Ponzetto-Zimmerman and Wolgemuth, 1984; Shaw *et al.,* 1983; Monk *et al.,* 1987and Hewlett and Reik 1991) that were obtained in various laboratories studied the protein composition of mouse liver in embryogenesis and adult hood using the SDS-PAGE analytical technique demonstrated the following major proteins :

1. (repetitive sequence protein (L1-line 1),Major urinary proteins (MUP's), Intercisternal A particle (IAP), Mouse interspersed family protein(MIF), Minor satellite protein(MIS), Major satellite protein(MJS).

2. With presumably some structural proteins such as (microtubular and membrane proteins) or some regulatory proteins (Comings and Tack, 1972).

Bird (2002) demonstrated that, the character of a cell was defined by its protein constituents resulted from specific patterns of gene expression.

Therefore, it was suggested that, lane (1) had represented the normal phenotype of the liver proteins, since the protein sample was isolated from the control mouse liver (which was not treated with any agent). However, the same phenotype was possibly detected in lane (3), where similar protein banding patterns were recognized. Therefore, it was probably suggested that, the gene expression was not affected by the treatment with (folic acid).

However, similar results were obtained by Fleming and Schilsky, (1992) in which they had reported that, folic acid was essential for DNA stability and therefore it did not affects the gene expression or the process of protein synthesis in the treated tissue.

Whereas, the protein constitution of the liver sample in lane(2) revealed that the presence of two polypeptide chains instead of the single band of probably (MUP's) recognized in others possibly indicated a case of protein subunit disorganization which might be a result of an effect on gene expression.

A role of MUP's was suggested by Bocskei *et al.,(*1992) exerted in the mating competence, so animals that showed difficulties in mating did not express it.

Moreover, it was found that, the absence of these proteins from the normal composition of the body was associated with lowering the body weight (Reik *et al.,* 1993). This case however, was noticed in the treated animals with (8mg/kg) of 5-azacytidine during the time course of the study suggesting that, treatment with 5-azacytidine had probably affected the gene expression in liver cells which resulted in differences in the migration rates between lanes (1, 2 and 3).

Thus, on the light of the obtained results in which a decrease in the body weights of treated mice males and females , histological abnormalities in the testes , functional and morphological abnormalities in the sperms , reduction in the fertility rate, reduction in the pregnancy out-come and teratogenic effects altogether had suggested that, 5-azacytidine might probably affected the gene expression through its hypomethylating activity which might be responsible for the results that were obtained in the study.

Chapter one

Literature review

1.1- Concepts of development:

Development is the process by which a multicellular organism arises, initially from a single cell, (the fertilized egg) (Twyman, 2001).

However, development is a progressive process so that, a simple embryo comprising few cell types organized in a crude pattern is gradually refined to generate a complex organism with many cell types showing highly detailed organization, and this gradual developmental strategy is known as epigenesist (Slack, 1997). In fact, development involves five major overlapping processes namely: growth, cell division, differentiation, pattern formation and morphogenesis (Twyman, 2001).

Figure (1.1) shows an overview of the mammalian development process according to (Twyman,2001).

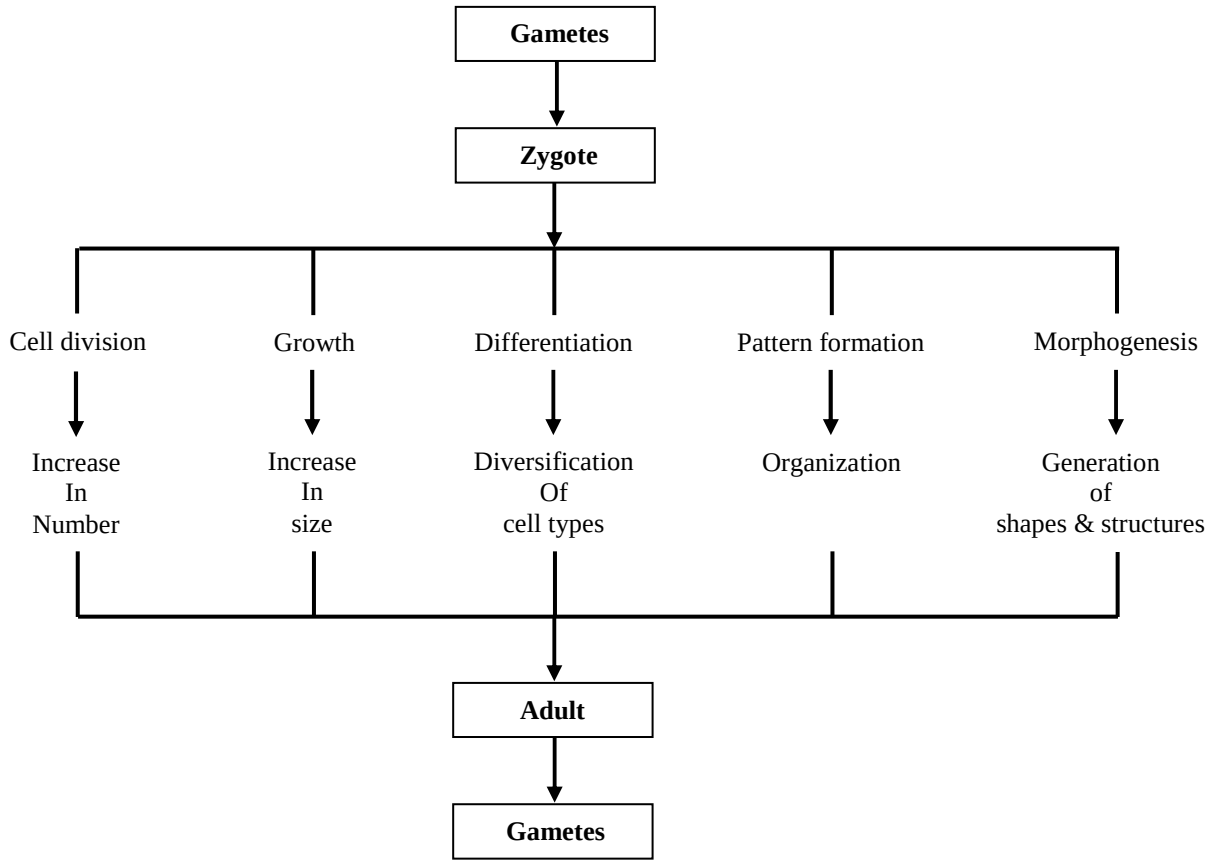


Figure (1.1): An overview of the development process

1.2- Genes in development:

All the processes that are essential for mammalian organism development were mediated ultimately by genes that coded for proteins, acting either directly or as enzymes to produce the necessary molecules (Twyman, 2001).Therefore, development were controlled to a large degree by gene expression (Russo *et al.*,1999). However, many evidences were obtained from several studies showed that, DNA methylation had contributed largely to the differential gene expression during development:-

Firstly, it was found that, the genome of the mammals undergoes global changes in methylation during early development followed by cell- specific changes in methylation patterns associated with the individual genes (Porter, 1998).In other wards, DNA methylation patterns were erased by a global wave of demethylation activity in the zygote, then, DNA methylation patterns were de novo generated in the epilast just prior to gastrulation (Kalthof, 1996).

Secondly, mammalian gene expression was in some cases inversely correlated with the amount of DNA methylation in the promoter, for example, during the differentiation of erythrocytes, the globin genes were expressed when their promoters were un methylated and vice versa (Porter,1998).

Thirdly, mammalian genes can be repressed or activated by adding or removing methyl groups, for example, in vitro full methylated transgenes were not expressed when they were introduced into a cell where methylation abolishes gene transcription (Jost and Saluz, 1993). Conversely incorporation of the cytosine analogue, the 5-azacytidine (major methylation inhibitor) reactivated the previously repressed genes (Morikawa *et al.*, 1982).

1.3- Development and epigenetic:

Epigenetic is the term applied to describe patterns of gene expression caused by mechanisms other than changes in the nucleotide sequence (Holliday, 1986; Henikoff and Matzke, 1997). A hallmark feature of

epigenetic regulation occurs principally at the level of transcription (Eden and Cedar, 1994).

In mammalian cells, the methylation state of the base cytosine can be inherited without altering the genetic material (Baylin, 1997).

This epigenetic form of inheritance generates patterns of DNA methylation that modulates overall genomic patterns of chromatin organization and gene expression (Wolffe and Matzke, 1999), revealing an interrelationship among DNA methylation, development and epigenetic regulation of gene expression (Hoekenga *et al.*, 2000).

It appears from several studies (Cedar, 1988; Weissbach, 1993; Baylin, 1997), that, DNA methylation served a variety of biological roles in mammal's development. Therefore, in order to understand the role played by the DNA methylation in development, this unique modification of DNA was more deeply reviewed.

1.4- DNA methylation:

Since its first recognition in 1948, the fifth base of mammalian DNA, 5-methylcytosine (5-mc) has generated much interest and considerable controversy during attempts to understand its significance (Weissbach, 1993).

DNA methylation in mammalians involve addition of a methyl group to the carbon ring through a reaction catalyzed by DNA methyltransferase in the context of the sequence 5-CG-3, which is also referred to as CPG dinucleotide (Antequera and Bird, 1993).DNA methylation is the only modification that occurs after DNA replication and what makes this modification unique is the fact that, it modifies the genome without changing the nucleotide sequence through the addition of methyl groups to the genome by an independent enzymatic machinery, (the DNA-methyltransferase), and removed from the DNA by an independent enzymatic machinery, the demethylases (Szyf,2000).

However, recent studies showing that genes expressing aberrant DNA methylation resulted in abnormal development and increased embryonic

lethality supported the critical role played by the DNA methylation in development (Yoder *et al.*, 1997).

Since enzymatic methylation of cytosine's in DNA was believed to have an effect on the physical properties as well as the structural features of the double helix.

Figure (1.2) shows the probable structural abnormalities of the double helix resulted from cytosine methylation (Jost and Saluz,1993).

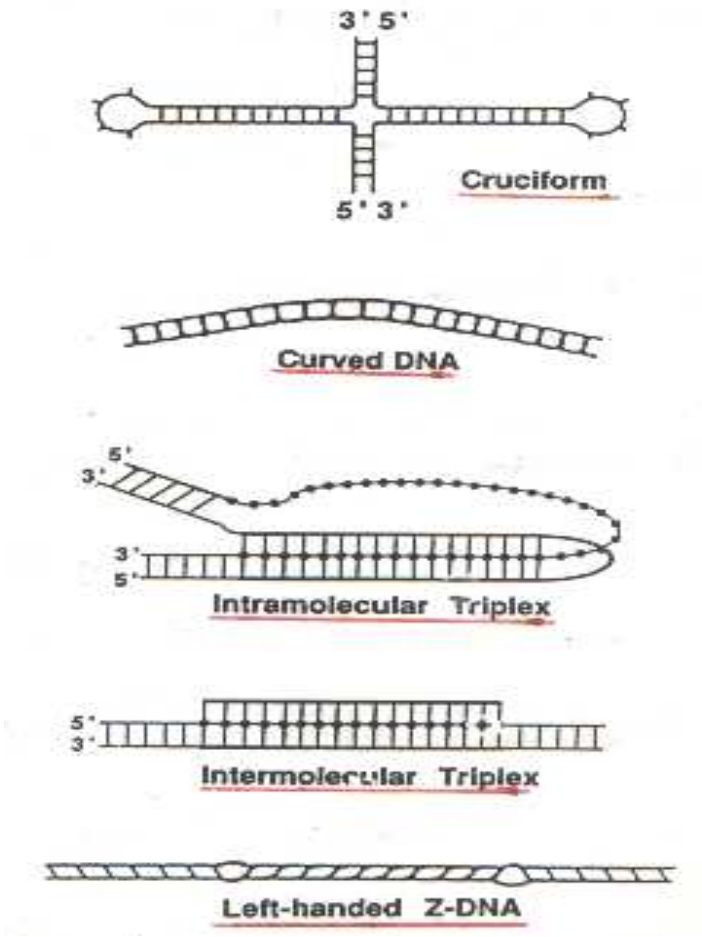


Figure (1.2): Structural alternatives to the right-handed-B-form of the DNA.

1.4.1- Distribution of methylated cytosines and CPG islands:

Mammalian genomes are not methylated uniformly but contain methylated regions interspersed with unmethylated domains (Bird, 1986).

During evolution, the dinucleotide COG has been progressively eliminated from the genome of higher eukaryotes and is present at only (5%-10%) of its predicted frequency (Antequera and Bird, 1993). Cytosine methylation appears to have played a major role in this process, because CPG sites lost represent the conversion through deamination of methylcytosines to thymines. (Szyf,2000).

Approximately (70%-80%) of the remaining CPG sites contain methylated cytosines in most vertebrates (Antequera and Bird, 1993).

These methylated regions are typical of the bulk chromatin that represents the late replicating DNA with its attendant histone composition and nucleasomal configuration as is relatively in accessible to transcription factors (Tazi and Bird, 1990). However, the CPG regions ranged in size from (0.5-5kb) and occurred at an average of every (100kb) along the genome (Antequera and Bird, 1993).

These regions were rich in (G + C) content which was greater than (60%) and were the target sites of the DNA-methyltransferase enzymes (Bird, 1986; Cross and Bird, 1995).

However, it was found that, the distribution of the CPG islands in the mammalian genome was as important as the role of DNA methyltransferase activity where two types of CPG regulatory regions were found (Antequera and Bird, 1993).

Genes who contained nonmethylated CPG islands in their promoter regions were usually called “housekeeping" genes that had a broad tissue pattern of expression (Antequera and Bird, 1993). While, the tissue – specific genes contained CPG islands which were variably methylated where the methylation state was inversely correlated with the transcriptional status of the genes (Cedar, 1988 and Bird, 1992).

Therefore, the lake of methylation of the CpG island within the promoter region of the gene was required for the transcription of the genes (Antequera and Bird, 1993). The main exceptions included the non-transcribed genes on

the inactive X-Chromosome (Heard *et al.*, 1997), and imprinted autosomal genes where one of the parental alleles was methylated (Baylin, 1997).

1.4.2- DNA methyltransferases:

The enzymes that transfer methyl groups to the cytosine ring, cytosine 5-methyltransferases, or DNA methyltransferases (DNA-Mtase) have been characterized in a number of eukaryotes (Gruenbaun *et al.*, 1972).

The first eukaryotic DNA – Mtase gene cloned from mouse almost a decade ago, which was referred to as Dnut1 (Bestor and Ingram, 1983).

This gene was found to be highly conserved among mammals with 5-methylcytosine (Jost and Salue, 1993).

One of the unique features of the mammalian DNMT1 enzyme was that, it has along amino-terminal domain, which contained multiple motifs that targeted the protein to sites of active DNA replication (Bestor and Ingram, 1983).

The mammalian DNMT1 enzyme displays an interesting developmental regulation in having regions unique either to male or female gametogenesis (Cheng, 1995). It was found that oocyte contained large a mounts of maternally stored DNMT1 enzyme, this enzyme shuttles after fertilization between cytoplasmic to the nuclear localization in relation to the wave of preimplantation demethylation and post implantation de novo methylation (Adams, 1995).

Mammalian DNmt1 methyltransferase has a high affinity for hemimethylated substrates but also capable of performing denovo methylation of unmethylated substrates in vitro (Leonhardt *et al*.,1992).

Evidences for an additional mammalian DNA- methyltransferase capable of denovo methylation came from the transfection experiments of (Li *et al*., 1992).These enzymes were named DNMT2, DNMT3a and DNMT3b.

1.4.3- Regulation of DNA methyllation in eukaryotic cell:

There are two basic types of normal methylation processes known in mammalian cells:-

First, is "de novo methylation" which is involved in the rearrangement of methylation pattern during embryogenesis and differentiation processes in adult cells (Monk, 1990; Razin and Cedar, 1993).

Recently a family of enzymes was described, containing two methyltransferases DNMT3a and DNMT3b which show the de novo methylation activity (Robertson *et al.,* 1999 and Xie *et al.,* 1999).

The homologous genes were identified in mouse (Hsieh, 1999) and targeting experiments showed that both DNMT3a and DNMT3b were essential for de novo methylation in humans and had no activity of maintenance methylation (Okano *et al.*, 1999).

The second methylation activity was the so – called “maintenance methylatioin” which is responsible for maintaining pattern once established (Bestor *et al.,* 1988).

The first mouse maintenance methylatransferase DNMT1 was described by Bestor et al., (1988). Then homologous enzymes were found in humans too (Yen *et al.*, 1992), in chickens (Tajima *et al.*, 1995).

The functional analysis of the enzyme showed that it has maintenance activity and is vitally important for embryonic development in the mouse (Li *et al.*, 1992).

During DNA replication, DNMT1 is located in the replication complex where it recognizes the normally methylated CpG sites in the parent strand and catalyses the addition of the methyl group in the corresponding CpG sites in the daughter strand (Lei *et al.*, 1996).

Active localization of the enzyme to sites of DNA replication in dividing cells may facilitate a maintenance role of DNMT1 (Leonhardt *et al.*, 1992).

1.4.4- DNA methylation and transcriptional repression:

A role for DNA methylation in the differential regulation of gene expression was hypothesized many years ago (Riggs, 1975; Holliday and Pugh, 1975).

The potential mechanism was suggested by a number of early observations in which site- specific cytosines methylation within or adjacent to genes was found to correlate with the transcriptional repression (Ginder and McGhee, 1979; Razin and Riggs, 1980).

Subsequently, this inverse relationship between cytosine methylation and transcription has been observed in a large number of genes (Razin and Cedar, 1991).

Numerous reports have shown the ability of the promoter DNA methylation to inhibit the transcription of a wide variety of genes in *in vitro* transfection assays, and in some cases, such methylation correspond to the inactive state of the gene under *in vivo* study (Bird, 1992; Razin and Cedar, 1991). However, Szyf (2000) had proposed three possible mechanisms to account for transcriptional repression by DNA methylation.

Figure (1.3) shows the events involved in each mechanism of DNA transcription repression.

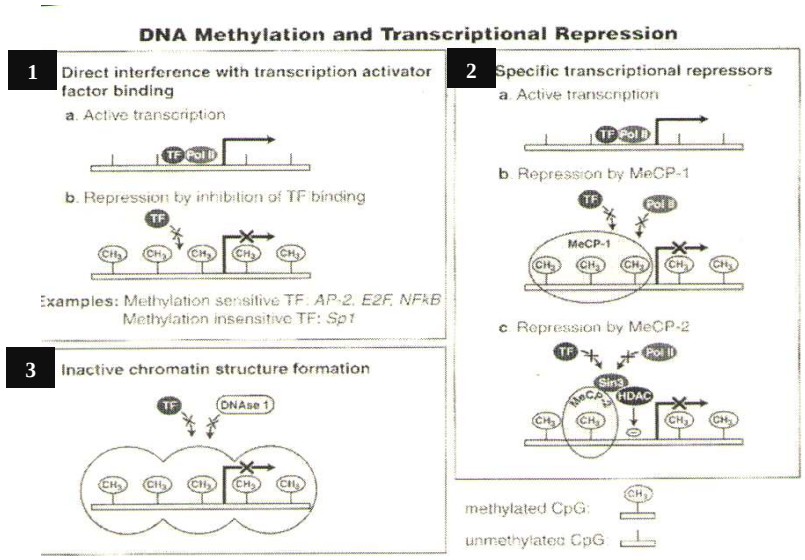


Figure (1.3): Proposed mechanisms of transcriptional repression mediated by cytosine methylation.

The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters (Tate and Bird, 1993).

Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP- dependent activator recognize sequences that contain CPG residues, and their binding to each has been shown to be inhibited by methylation (Tate and Bird, 1993).

In contrast, other transcription factors, such as (SP1) was shown to be insensitive to the methylation of their binding sites (Rountree and Solker, 1997).

A second potential mechanism for methylation induced silencing is through the direct binding of specific transcriptional repressors to methylated DNA (Meehan *et al.,* 1989).

Two such factors, MeCP-1 and MeCP-2 (methyl cytosine binding proteins 1 and 2), have been identified and shown to bind to methylated CPG residues in any sequence context (Boys and Bird, 1997).

A third mechanism by which methylation may mediate transcriptional repression is by altering chromatin structure (Razin and Riggs, 1980).

Experiments using microinjection of certain methylated and nonmethylated gene templates into nuclei have shown that methylation inhibits transcription only after chromatin was assembled (Razin and Cedar, 1991).

Transcriptional silencing by DNA methylation appears to be a more common mechanism among tissue – specific genes in that genes usually required specific transcriptional factors for their gene activity (Kass *et al.*, 1997).

1.4.5- DNA demethylation during development and tissue-specific differentiation:

A critical aspect of the overall regulatory role of DNA methylation is the process of demethylation (Sullivan *et al.,* 1989). Using methylation sensitive restriction enzymes to monitor the general level of DNA methylation, it was shown that, during early development, a dramatic reduction in methylation levels occurs in the preimplantation embryo (Monk *et al.,* 1987). This was followed by a wave of de novo methylation involving most CPG residues but leaving the CPG islands unmethylated at the time of implantation (Kafri, *et al.,* 1992). After implantation, most of the genomic DNA is methylated, whereas tissue – specific genes undergo demethylation in their tissue – or gene – specific removal of methylcytosines from DNA must exist (Szyf,2000).

Demethylation could be a passive process such as the inhibition of methylation after DNA replication (Razin and Riggs, 1980). On the other hand , Kafri (1992) had reported on the existence of an active demethylation process not involving DNA replication in mammalian cells has been supported by a number of observations:-

In transformation assays cells, a DNA methyltransferase inhibitor was shown to have a demethylation activity that caused the differentiation of murine fibroblast cells to myoblasts, adipocyte and chondrocytes (Antequera and Bird,1993).

These experiments ultimately led to the identification and doing of the MyoD gene which showed that treatment with 5-azacytidine caused the demethylation of the MyoD "CPG" is lands and was associated with the activation of the MyoD gene expression (Rudicki and Jaenisch, 1995). In addition, it become clear that, the widespread methylation of CpG islands was an aberrant phenomenon associated with prolonged *in vitro* passage of cells what should be noted in studies documenting a role for DNA methylation in

the regulation of gene expression and aging of cells in culture (Russo *et al.*, 1999).

Furthermore, several studies showed that, changes in DNA methylation patterns in cancer cells parallel those seen in tissue culture and aging cells when compared to normal cells (Laird and Jaenisch, 1996). It was found that cancer cells had reduced global levels of DNA methylation often accompanied by an increase in DNA –methyltransferases activity (Kautainen and Jones, 1986 and Issa *et al.*, 1993) with a localized increase of DNA methylation in particular at specific CPG islands (Laird and Jaenisch, 1996).

Figure (1.4) shows examples of possible mechanisms where DNA methylation could induce cancer.

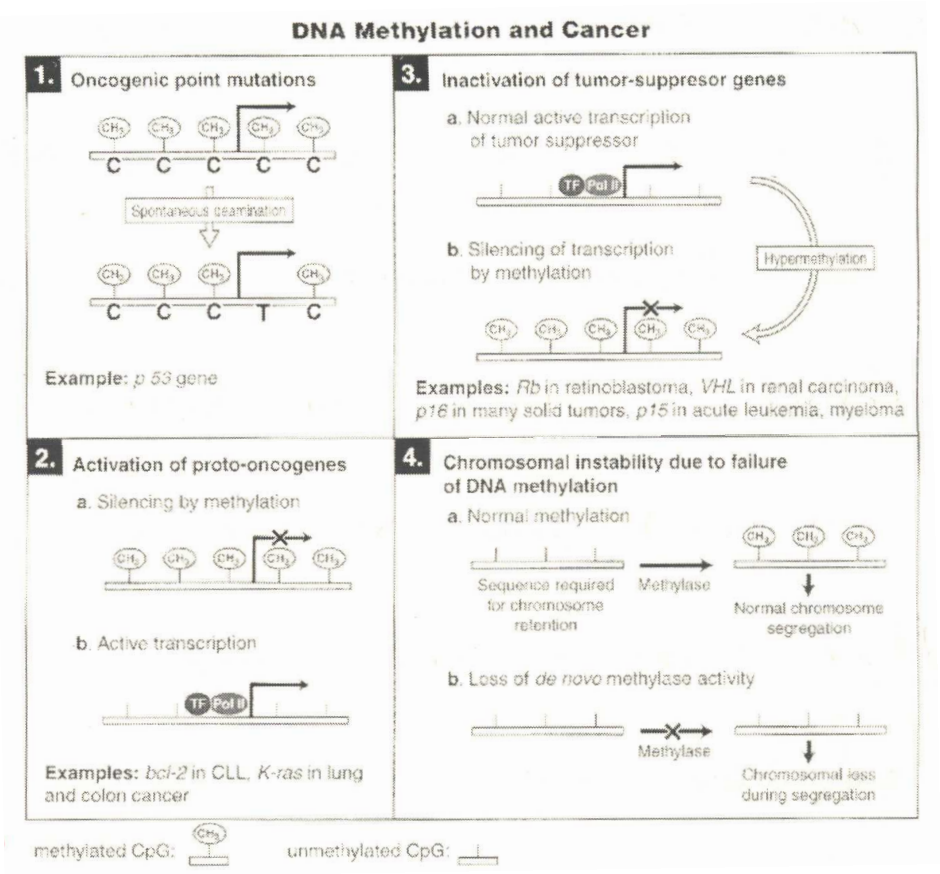


Figure (1.4): Models for the different mechanisms through which cytosine methylation can promote oncogenesis.

1.4.6- Clinical implication and applications of DNA methylation:

DNA methylation patterns are likely to become of increasing importance in the management of cancer patients in the near future (Bird, 1996). Already several clinical trails using agents targeted against DNMTS have been completed or under way (Costello and Plass, 2001). In addition, DNA methylation shows great promise as a marker for the early detection of cancer, and DNA methylation patterns might be of use in determining patient prognosis (Strathdee and Brown, 2002).

1.4.6.1- Therapeutics targeting DNA methylation:

Methylation probably causes the inactivation of numerous genes that are important in the development of most or all tumour types; thus, inhibition of DNA methylation and consequent reactivation of these genes is an attractive avenue for the development of novel therapeutics (Strathdee and Brown, 2002). This strategy is particularly appealing because, in normal cells, these genes are not normally regulated by DNA methylation and therefore, the toxicity of inhibitions of DNA methylation to non-cancer tissues could potentially be well below that seen with conventional cytotoxic anti-cancer agents (Jones, 1985b).

However, as yet, few effective inhibitors of DNMTs were known ; the two closely related drugs 5 - azacytidine and 2 – deoxy – 5 - azacytidine (Stratheeda and Brown, 2002), have long been used experimentally to inhibit DNA methylation in tissue culture and have been shown to re-activate numerous methylation – silenced genes (Jones, 1982; Jones, 1985a). In addition, 5-azacytidine has also been shown to induce cell differentiation (Jones, 1985a), and has been used to treat a number of haematopoietic disorders (Pino and Zagonel, 1993). However, the fundamental cancer treatment “the chemotherapy” have long been known to have a multitude of short and long term effects (Arnon *et al.*, 2001).

Witt and Bishop(1996) showed that these drugs could be mutagenic to somatic cells, causing gene mutation, chromosomal rearrangements, breaks and aneuploids.

With a growing population of young cancer survivors (Boring *et al.*, 1994), significant concerns have been raised regarding the adverse effects of these treatments on the off spring of the treated individuals. That include off springs conceived after completion of treatment, damage to the genetic composition of the human germ cell might influence fertilization, increase in the rate of abortion, or cause malformations in children conceived by men or women previously exposed to cancer treatment (Kennedy *et al.*, 1993).

1.4.6.2- Inhibitors of DNA methylation:

1.4.6.2.1- 5-azacytidine:

The first class of DNA inhibitors included nucleoside analogs that were phosphorylated by cellular kinases, incorporated into the DNA and inhibited methylation. (Szyf, 1996). Figure (1.5) shows examples of nucleoside analogs of DNA inhibitors (Goffin and Esinhauer, 2002).

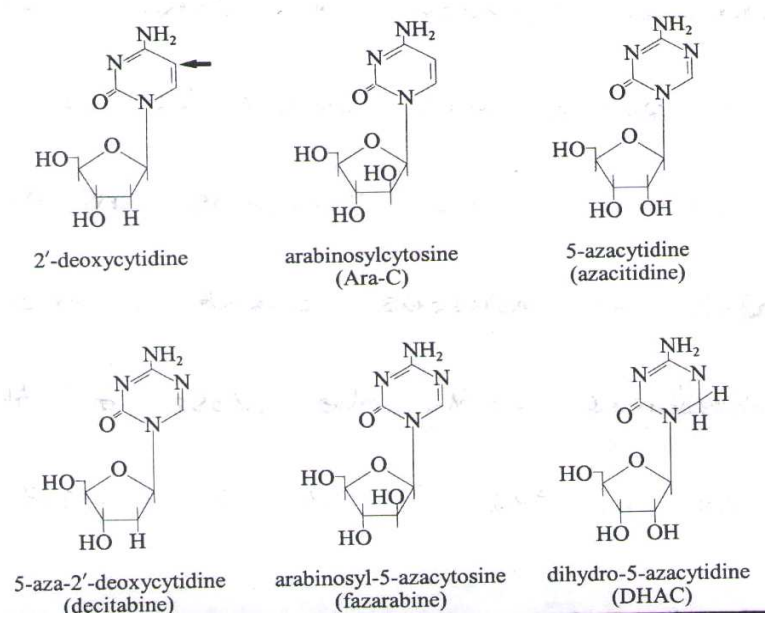


Figure (1.5): Inhibitors of DNA methyltransferases.

The classic inhibitor of DNMTs is 5-aza-cytidine (5-aza-c) Which is a ring analog of the natural pyrimidine nucleoside cytidine, differing by a nitrogen instead of the number 5- carbon (Bergy and Herr, 1966).

This analog was originally developed for use as a chemotherapeutic agent (Wesley and Cihak, 1978) and is still used as part of the treatment regimen for acute myelogenous leukemia (Goffin and Esinhauer, 2002).

The S-triazene ring structure of 5-azacytidine is unstable in aqueous solution and is rapidly hydrolyzed with the loss of the carbon atom in the 6-position (Notari and De-Young, 1975).

5-azacutidine enters cells where it is phosphorylated by uridine- cytidine kinas and subsequently was incorporated into both the RNA and DNA of treated cells (Li *et al.,* 1970). Taylor found that greater than (85%) of incorporated 5-azacytidine has an intact ring structure in DNA, implying that the triazine ring might be relatively stable within the macromolecule.

However, 5-azacytidine induced genotoxic effect without directly damaging the DNA but, it causes a change in the DNA conformation (Stopper, 1997).

Since DNA methylation was associated with gene expression, 5-azacytidine exerted its effects through re – expression of silenced genes (Bender *et al.,* 1998).

Azacytidine could induce cellular differentiation, expression of several proteins, re expression of antigens, and erythrocyte differentiation (Taylor and Jones, 1979; Nomparler *et al.,* 1985; and Atadia, 1993). Exposure of murine 10T1/ 2 embryonic fibroblasts to 5-azacytidine induced formation of mature muscle cells or adipocytes (Taylor and Jones, 1979). This finding suggested the importance of a modification in the 5 position of the pyrimidine ring for the induction of cellular differentiation (Santine *et al.,* 2001). *In vitro,* inhibition of DNA methylation by suppressed growth of tumour cell lines but not normal fibroblasts, and the effect of

5-azacytidine on the proliferation of L1210 leukemia cells was related to does and duration of exposure. (Bender *et al.*,1998 and Covey and Zaharko, 1984).

In addition, 5- azacytidine was also tested for carcinogenicity by intraperitoneal injection in four studies in mice and it was found to accelerate the development of leukemia, incidence of lymphoid neoplasm, lung adenomas and testicular tumors (IARC, 1990).

1.4.6.2.2- Methotrexate:

The antimetabolites are synthetic analogues of normal metabolites that act by competition (Laurence *et al.,* 1997), and the interest in antimetabolite cancer chemotherapy began in the late 1940S with the demonstration by Farber and colleagues that the antifolate aminopterin (2, 4–diamino, 4-deoxyfolic acid) could induce remissions in pediatric acute leukemia. Later, antimetabolites of specific nutrients and their metabolites found great applicability as chemotherapeutic agents against cancer such as methotrexate (Poirier *et al.,* 2003). This is the most widely used antifolate in medical oncology (Takiomoti, 1996). This N – methyl derivative of aminopterin has documented activity in a cute leukemia, non – Hodgkin s lymphoma, breast cancer, head and neck cancer, choriocarcinoma, estrogenic sarcoma and bladder cancer (Fleming and Schilsky, 1992). Antifolates are also widely used in the treatment of nonmalignant disease such as rheumatoid arthritis, bacterial and plasmodia infections (Fleisher, 1993), and in the opportunistic infections associated with AIDS (Takimoto and Allegra, 1995).

The anti-cancer drug methotrexate differs from the essential vitamin, folic acid, by the substitution of an amino group for a hydroxyl at the u- position of the pteridine ring.

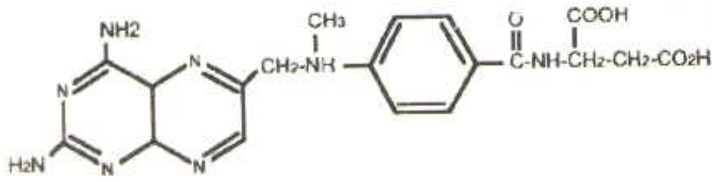


Figure (1.6): Structure of methotrexate.

This minor structural alteration changes the normal substrate into a tight – binding inhibitor of dihydrofolate reductase (DHFR) (Neill, 1998) the enzyme principally responsible for the maintenance of the intracellular reduced folate pool (Li *et al.,* 1992). Reduced forms of folic acid called tetrahydrofolates are essential cofactors which serve as single carbon atom donors in the enzymatic synthesis of thymidylate and purine nucleotides (Chu *et al.,* 1993).

Tetrahydrofolates are only biologically active in their fully reduced forms (Takimoto, 1996). Methotrexate is considered as potent competitive inhibitor of dihydrofolate reductase since, tetrahydrofolate is oxidized in biosynthetic reactions and the presence of methotrexate prevents its reduction (Poirier, *et al.,* 2003). He resulting starvation of reduced folate limits methylation reactions including the synthesis of thymidine, methionine and purines (Neill, 1998).

It might also influence events such as polyamine synthesis and epigenetic marking of DNA by methylation through limiting the production of the methyl donor S-adenyl – methionine (Alexiou and Leese, 1992).

Since thymidine does not accumulate in cells, an important intermediate consequence of methotrexate treatment is the inhibition of cell division due to inhibition of DNA synthesis, secondary to thymidine starvation (Neill, 1998). Thus the inhibition of DNA synthesis by methotrexate is a multifactor process which results from both the partial depletion of intracellular reduced folates

and from the direct inhibition of folate – dependent enzymes (Poirier *et al*., 2003).

In experimental studies, methotrexate appeared to exert carcinogenic activity towards the mammary glands of male rats (Rogers *et al*., 1990).

In one study, methotrexate has caused, significant but moderate, pancreatic toxicity in mice (Woolley, 1983). Methotrexate is a well-known teratogen in both humans and experimental animals (Bawle *et al*., 1998 and DeSesso and Jordan, 1977) with cleft palates and neural tube defect in embryos of treated females (Elmazar and Nau, 1992).

On the other hand, methotrexate treatment decreased the extent of DNA methylation in the brains and livers of rats (Poirer *et al.,* 2003). Therefore, methotrexate, like other chemotherapeutics inhibitors of DNA synthesis, induced DNA hypomethylation in cells (Kim *et al.,* 1996).

1.4.6.3 – Methyl Donors:

Many micronutrients and vitamins are critical for DNA synthesis / repair and maintenance of DNA methylation patterns (Fenech and Ferhus2001).

Folate has been most extensively investigated in this regard for its Unique function as methyl donor for nucleotide synthesis and biological methylation (Frisco and Choi, 2002). Folate is a water soluble B vitamin, that is essential for the synthesis of S-adenosylmethionine (SAM), a compound a compound that serves as the methyl donor for over (100) biochemical reaction, including the DNA methylation (Kim, 1992). It is in this manner that folate modulates DNA methylation (Figure 1.7).

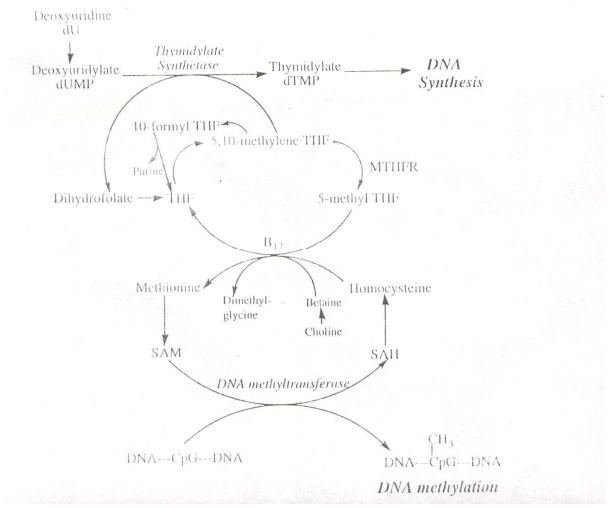


Figure (1.7): Scheme of folate metabolism.

As shown in this figure , methylenetetrahydro – folate reductase (MTHFR) is a critical enzyme in folate metabolism, Its product 5-methyltera-hydrofolate (5-methyl THF), is the predominant form of folate in plasma, whereas , 5,10-methylene THF, is found mainly intracellular (Kim , 1992).

5-MethylThf provides the methyl group for de novo methionine synthesis and DNA methylation (Wagner, 1995), whereas the substrate for MTHFR, 5,10-methylene THF, is required for conversion of deoxyuridylate to thymidylate and can be oxidized to 10-formyl THF for de novo purine synthesis (Czeizel and Dudas, 1997). Therefore, 5, 10-methylene THF is critical in DNA biosynthesis as well as maintaining deoxynucleotide pool balance (Wagner, 1995). Consequently, a deficiency of folate in tissues with rapidly replicating cell results in ineffective DNA synthesis, resulting in reduced cell proliferation, impaired cellular physiology and abnormal cytologic morphology (Kim, 1992).

This biochemical function of folate has been utilized in the area of how folate modulates cell proliferation in the process of carcinogenesis (Mason and Levesque, 1996).

This leads to ineffective DNA synthesis, resulting in the inhibition of tumor growth. Indeed, this has been the basis for antitumor therapy using a number of antifolate agents, including methotrexate and 5-fluorouracil (Kamen, 1997). And (Bertino, 1997). However, cell culture and animal studies showed that deficiency of folate induces description of DNA as well as alteration in DNA methylation status (Robertson and Wolffe, 2000).

Animal models of methyl deficiency demonstrated stronger cause-and – effect relationship than did studies using cell cultures (Frisco and choi, 2002). Such observations implied that, the adverse effects of inadequate folate status on DNA metabolism are mostly due to the impairment of methyl supply (Ames, 2001). Animals fed diets deficient in methyl donors (folate, chlorine and methionine) have hypomethylated DNA. (Selhub *et al.*, 1991).

Zeisel and Blusztajn (1994) demonstrated that, these changes occur not only in global methylation, but also in the methylation of specific genes.

Mouse liver tumorigenesis induced by a chalino devoid, methionine – deficient died was associated with the hypomethylation of c-Has – ras and raf oncogenessis (Lu *et al.,* 2001). Folate deficiency is associated with human gastric carcinogenesis, neural tube defect in the fetus (Slattery *et al.,* 1997) (Alonso and Moreiras, 1996).

These findings suggested that, the interaction between nutritional statuses with a genetic polymorphism can modulate gene expression through DNA methylation, especially when such polymorphism limits the methyl supply (Laurence *et al.,* 1997).

DNA methylation, both genome – wide and gene - specific, is of particular interest for the study of cancer, aging and other conditions related to cell – cycle regulation and tissue – specific differentiation, because it affect gene expression without permanent alterations in DNA sequence such as mutations or allele deletions (Frisco and Choi ,2002).

Understanding the patterns of DNA methylation through the interaction with nutrients is fundamental, not only to provide pathophysiological

explanations for the development of certain diseases, but also to improve the knowledge of possible prevention strategies by modifying the nutritional status in risk – populations (Niculescu and Zeisel, 2002).

1.4.7- Studying DNA methylation by means of animal cell culture:

Animal cell culture is defined as growth of cells dissociated from the parent tissue by spontaneous migration or mechanical or enzymatic dispersal (Doyle *et al.*, 1990). It has become an indispensable technology in many branches of the life sciences where it provides the basis for studying the regulation of cell proliferation, differentiation and cytotoxic measurements (Davis, 1994).

Two major advantages were implied in this technology the control of the physicochemical environment (pH, temperature, osmotic pressure, O_2 and CO_2 tension), and the physiological conditions, which might be kept constant by supplementation of the medium with serum or other poorly defined constituents (Honn *et al.*, 1975).

During propagation, a precursor cell type will tend to predominate, rather than a differentiated cell; consequently a cell culture may appear to be heterogenous, as some cultures such as epidermal keratinocytes (Freshney, 1994). While, other cultures, such as murine fibroblasts, contain a relatively uniform population of proliferating cells at low densities of a bout (10^4 cell/cm^2) (Masters ,2000).

However, the choice of the cell type, the source of tissue and whether it is normal or neoplastic were all dependent on the question being asked about the ability to survive and proliferate, differentiate and the interactive nature of growth control (Masters, 2000).

1.4.7.1- Techniques of mammalian cell culture:

The manner in which mammalian cells are cultivated varies widely, depending not only on the requirement of the cells but also upon the applications in culture (Barnes and Sato, 1980). Mammalian cells, however, are more sensitive to their environment for being large cells not protected by a hardy cell wall, and its nutritional requirements are more complex (Freshney, 1983). The mammalian cell in vivo is not free living; it is dependent upon a circulatory system that has developed to ensure a precisely regulated homeostatic environment (Maramorosch, 1983).

There is an abundance of different cell type that appear to have special requirements for growth and function in vitro , but , most normal animal cells require specially treated and charged surfaces for selective attachment as a monolayer and for growth (Kahn *et al.*, 1964). On the other hand, the medium is probably the most important factor in the maintenance and growth of cells (Freshney, 1983).

Kruse and Paterson (1973) demonstrated that there are no universal guide lines for the selection of medium, serum, or growth factors for a given cell type. For most cell lines, it was recommended that cells be kept in the medium to which they are accustomed (Maramorsch, 1983).

However, growth curves can be done for each set variables, determining lag time, generation time, and cell density at the stationary phase (Kruse and Paterson, 1973).

Since there are numerous media used in mammalian cell culture, the medium environment must satisfy more than just the nutritional requirements of cells (Barnes and Sato, 1980).

Proper osmotic pressure is one parameter that is maintained by the appropriate concentrations of salts and glucose where cells from different species vary in their ionic requirement (Freshney, 1983).

While, the pH of most biological fluids is maintained near neutrality, most cells survive at a pH range between (6.8 and 7.6), however, the pH in most

media is controlled by a combination of dissolved gasses (Sodium bicarbonate buffer system) and product of metabolism by the cell (especially lactic acid) (Barnes and Sato,1980). In addition, a source of carbohydrate is necessary in cell culture medium to supply an energy source and the most common source of carbohydrate is glucose , but other monosaccharide (for example, galactose) can also be used

(Feder and Tolbert, 1983). Moreover, amino acids are required for cell growth and function; and most animal cells have a specific requirement for 13 amino acid (Arginine, Cysteine, Glutamine, Histidine, Isoleucine, Leucine, Lysine, methionine, phenylalanine, threonine, trytophane, tyrosine, and valine) and only the L forms are utilized (Freshney , 1983).

On the other hand, vitamins are used as cofactors in cell metabolic functions. In certain cell types, such as bone cells, ascorbic acid is important for growth and function (Binderman *et al.*, 1974).

Peehl and Ham (1980) showed that, vitamins and most minerals required by cells in culture are provided by the serum in addition to the growth factors and proteins are a major component of serum, they may serve as carriers for minerals, fatty acids and hormones (Barnes and Sato, 1980).

As with media, there are no universal guidelines for the addition of sera for a specific cell type (Feder and Tolbert, 1983).

Generally, trial and error methods are employed to select the serum best suited for the growth and function of cells (Maramorosch, 1983).

1.4.7.2- Types of cultivated cells:

1.4.7.2.1- Fibroblast cells:

Fibroblast cells are the most common cells present in tendons and ligaments, they also occur in the embryonic connective tissues (Saladin, 1998). Fibroconnective tissues are generally regarded as the weeds of the tissue culture garden (Freshney, 1994). They survive most mechanical and

enzymatic explanation techniques and may be cultured in many of the simplest media, such as Eagle‘s basal medium (Honn *et al.,* 1975).

Fibroblast cells have been isolated from many different tissues such as the vascular connective tissues of the blood vessels (Goldberg, 1977), from embryonic and a dult tissues (Raff *et al.,* 1979).

Cultures of hamster, mouse and chick embryonic fibroblasts could be prepared by many techniques such as, (Mechanical desegregation or by enzymatic desegregation using enzymes such as cold or warm trypsin or collagenase (Freshney, 1994). Generally, fibroblast cells were characterized as large, flat cells with slender branches; in tissue sections, they often appear tapered at the ends (Saladin, 1998).

Therefore, they produce the fibers and ground substance that together form the matrix connective tissue. Masters (2000) demonstrated that, human, hamster and chick embryonic fibroblast cell cultures were morphologically distinct from mouse fibroblast cells, as they assume spindle – shape at confluence producing a characteristic parallel assays of cells distinct from the pavement – like appearance of mouse fibroblasts.

However, the conformation of fibroblast like cells in vitro required the identification of the appropriate markers such as collagen type I and Thy I antigen (Freshney, 1994).

Kruse and Paterson (1973) reported that, once a culture was initiated, it needed a periodic medium change followed eventually by subculture if the cells were proliferating rapidly. However, the first subculture gives rise to "a secondary subculture" and the secondary to “a tertiary subculture" and so on, which referred to by the term “the passage number".

Doyle *et al.,* (1990) showed that, normal fibroblast cell lines behaved with a limited culture fife – span (finite cell lines) and were grown for a limited number of passages (6-12 passages). In addition, Davis (1994) showed that, normal fibroblast cells were characterized by the “contact inhibition

"phenomenon which was designated by motility cessation of cells, membrane ruffling and reduction of growth when confluence was reached.

Holly *et al.*, (1978) noticed that, the reduction of normal cells growth after confluence was not solely due to contact of each cell with the other, but it also involved reduced cell spreading, depletion of nutrients and, particularly the growth factors in medium. However, fibroblast cell cultures could be transformed spontaneously or by viruses or chemicals so that, they loss the property of density limitation and reach to higher cell density in the plateau phase than their normal counter parts (Watermark, 1974).

Aaronson and his colleges (1970) showed that, transformed mouse 3T3fibroblast cells and human fibrosarcoma cells were able to form colonies on confluent monolayer.

On the other hand, Kuriharcuch and Green (1978) decaled that, it was possible to transfer cells from one lineage to another under certain conditions. Such transdifferentiation was confirmed in the transformed cells such as mouse 3T3 fibroblast cell line that was inducing by 5-azacytidine to differentiate into mycocytes, adipocytes and chondrocytes (Jones, 1979). While, this case has been rarely confirmed for the normal fibroblasts (Masters, 2000). Table (1.1) implies some of the transformed cells properties (Freshney, 1994).

Table (1.1): Properties of transformed cells

Characteristics	Transformed cells
Growth characteristics	Immortal Loss of contact inhibition high plating efficiency low serum requirement growth factor independent
Genetic properties	High spontaneous mutation rate Aneuploid, Heteroploid Overexpressed oncogenes deleted suppressor genes
Structure alterations	Modified actin cytoskeleton loss of cell-surface fibronectin increased lectin agglutination modified extracellular matrix altered cell adhesion molecules disruption in polarity
Neoplastic properties	Tumorigenic and Invasive increased protease secretion
Examples	Mouse 3T3 cell line, 10T1/2 fibroblast cell line

1.4.7.2.2- Plasmacytomas:

Plasmacytomas are solid tumors of malignant plasma cells similar to those found in the bone marrow (Wood and Philips, 2003).

While, plasma cells are the fully mature end-stage cell in the clonal B-cell development and their chief function is to secrete immunoglobulin (Ho *et al.*, 1986), plasmacytomas appear to arise from cells specialized in such away that, they can produce only a single molecular type of immunoglobulin (Byrde *et al.*, 1997).

Thus, plasmacytomas are of monoclonal origin on the basis of the homogeneous immunoglobulin that each tumour produces (Anderson, 1970).

Spontaneous plasmacytomas in mice occur rarely, but have been encountered several times in many strains (Potter, 1986).

Dunn (1957) described Spontaneous plasmacytomas that were developed as inflammatory lesions underlying ileocecal mucosal ulcer in aged (3H mice strain, spontaneous plasmacytomas were developed also in the bone marrow (57BL / KaLwRij mice strain and were indicated at high incidence in many other strains of mice such as (BALB / c; NZB; DBA / 2 ; A / He; A / LN; C57BL / He and SWR). (Goldstein *et al.,* 1966; Yamada *et al.,* 1969 and Rapaille *et al.,* 1993).

However, Rapaille *et al.,* (1993) demonstrated that, the most commonly used induction system of plasmacytomas in mouse is the peritoneal plasmogenesis, which is evoked by the intraperitoneal introduction of poorly metabolized materials such as paraffin oils (Potter, 1997), or the pure alkanes that are the components of these oils such as, pristine (2,6,10,14-tetramethylpentadecane) (Anderson, 1970), silicon gel (Potter *et al.,* 1992) and solid Lucite objects (Anderson, 1970). These agents induce chronic inflammatory reactive tissues in peritoneal connective tissues and are the sites where plasmacytomas develop (Potter *et al.,* 1985).

Four biological features of the peritoneal plasmacytogenesis are the:-

1. Immortalization in vitro where they can produce continuous cell lines with an infinite life-span (Potter, 1997).
2. Lossof contact inhibition and grow on confluent monolayers (Anderson *et al.,* 1970).
3. Anchorage independence as a result of cell surface modifications (Farhangi, 1986).
4. Deficiency in hypoxanthine guanine phosphoribosyl transferase (HPRT-ve), which makes the cells resistant to hypoxanthine, aminopterin, and thymidine (HAT) medium (Dean and Danford 1984).

Morphologically, plasmacytomas are usually rounded cells of different sizes when they are single; while monolayers composed of different sizes of rounded cells and short epitheloid cells which are adhered to the tissue culture flask surface, and upon confluence, the cells were more rounded and loosely attached to the vessel surface (Rapaille *et al.,* 1993). However, tumour transplantable cell lines such as, plasmacytomas are usually quite stable and maintain the continuous production throughout many generations (Farhangi, 1986), for example, the oldest immunoglobulin – producing tumour cell line (Myeloma cell line X5563) has been in nearly continuous passage since 1957 and is still producing the immunoglobulin (Potter *et al.,* 1985).

Plasmacytomas have been most useful in providing a source for large quantities of homogeneous immunoglobulin which can be isolated from the serum, ascites or urine of the syngenic mice transplanted with these tumors (Anderson, 1970). Silva *et al.,* (1997) demonstrated that the use of plasmacytomas as an example of malignant cells is of particular experimental and theoretical interest because these neoplastic cells are successfully adapted to the in vitro conditions and could be sub cultured for more than sixty passages (Farhangi, 1986). In addition, plasmacytomas provide an experimental model system for the screening of anticancer drugs (Rapaille *et al.,* 1993).

While all drugs in medical oncology have intracellular targets that are usually well characterized, thus, "target therapy" is reserved for agents that have been selected or, in some cases engineered, to interfere with a specific molecular target that is central to malignant behavior (Wood and Philips, 2003). The best example of targeted therapy came from agents that interfere with enzymes in the single transduction pathways that could disrupt normal regulatory signals causing malignant behavior such as (5-azacytidine) (Wood and Philips, 2003). As the dynamic properties of cell culture (Proliferation, differentiation and nutrient utilization) are sometimes difficult to control, and the complexity of cell interactions found in vivo can be difficult to recreate in

vitro, there have been numerous attempts to use laboratory animals to study the effects on the original tissue or organs (Masters, 2000).

1.4.8- Studying DNA methylation in vivo using the white mouse:

Many investigators used the white mouse as their model organism to study the carcinogenic and mutagenic effects of many drugs on various organs (Kevin, 1973 and Kuroda, 1997).

Laboratory mice were used as active in vivo and in vitro systems that resembled the human on many levels especially on the genetic one where a simple similarity was noticed between the human and mouse set chromosomes (Goodenough, 1978). Therefore, the fine results obtained from studying those laboratory animals were relayed on for humans (Tic and Levtt, 1985; Preston *et al.,* 1987). Furthermore, the development of transgenic mouse technology, together with the well- established genetic background of the mouse has added further impetus to the selection of mouse as a favorite experimental animal (Beddington, 1992).

Although, almost all mouse organs were subjected to extensive investigations by many workers (Peat *et al.,* 1992 and Kuroda, 1979). The complexity and functional importance of the mouse (male and female) reproductive organs, (the physiological and cytological processes taking place during their development) had attracted much attention (Clermont, 1972).

1.4.8.1- Mouse male reproductive system:

1.4.8.1.1- Testis:

Germ cells migrate to the gonadal ridge and were carried into the medulla by the primary sex cords, formed from the coelomic epithelium, to form the germinal epithelium of the somniferous tubules (Hafez, 1987).

In most strain of mice, fetal testes are recognizable by the 13 days of gestation (Whittingham and Wood, 1983). Testes are oval organs (Snell, 1984). The outer part consists of a thick, white connective tissue capsule.

Extensions of the capsule project into the interior of the testis and divide each testis into lobules.

The lobules contain seminiferous tubules, in which sperm cells develop. (Seeley *et al.*, 1996). Delicate connective tissue surrounding the tubules contains clusters of endocrine cells called "interstitial cells" or cells of "Leydig" which secrete testosterone (Snell, 1984). The seminiferous tubules empty into a tubular network called the "rete testis". The rete testis empties into (15 to 20) tubules called efferent ductules, and the efferent ductules exit the testis into the epididymis where the sperm cells continue their maturation and develop the capacity to swim and the ability to bind to the oocyte (Seeley *et al.*, 1996).

1.4.8.1.2-Spermatogenesis:

Spermatogenesis is the formation of sperm cells (Snell, 1984). Before puberty, the testes remain relatively simple and unchanged from the time of their initial development. At the time of puberty, the interstitial cells increase in number and size, the seminiferous tubules enlarge, and spermatogenesis begins (Seeley *et al.*, 1996). The seminiferous tubules contain germ cells and Sertoli cells (Kelly *et al.*, 1984). Sertoli cells are large cells that extend from the periphery to the lumen of the seminiferous tubule; they nourish the germ cells and produce a number of hormones (Hafez, 1987).

Germ cells are scattered between the Sertoli cells (Snell, 1984). The most peripheral cells are spermatogonia, which divided through mitosis; some daughter cells produced from these mitotic divisions remains spermatogonia and continue to divide by mitosis, other daughter cells from primary spermatocytes, which divide by meiosis (Seeley *et al.*, 1996).

A primary spermatocyte contains (40) chromatides (Zanefeld, 1978). Each primary spermatocyte passes through the first meiotic division to produce secondary spermatocytes, each secondary spermatocytes undergoes a second meiotic division to produce two smaller sex cells called "spermatids", each having (20) chromosomes (Orlando *et al.*, 1985).

After the second meiotic division, the spermatids undergo major structural changes to form sperm cells (Eddy, 1988). Much of the cytoplasm of the spermatids is eliminated, and each spermatic develops a head, middle-piece, and flagellum (tail) to become a sperm cell or spermatozoon (Garner and Hafez, 2000). The nucleus of the cells, just anterior to the nucleus is the vesicle called the "acrosome", which contains enzymes that are released during the process of fertilization (Seeley *et al.,* 1996).

1.4.8.1.3- Hormonal control of spermatogenesis:

The process of spermatogenesis takes place at a certain time which is characteristic for that particular species (Clermont, 1972). Hormones can alter the number of spermatogonia advancing through spermatogenesis, but they do not affect the rate at which the process occurs (Findlay, 1984).

Comhair (1988), reported a two axis's hormonal control of the spermatogenesis process involved:- (1) Hypothalamic- hypophysial-seminiferous tubule and (2) Hypothalamic – hypophysial – Leydig cells achieved through a number of hormones namely (the luteinizing hormone (LH), the follicle stimulating hormone (FSH) and testosterone.

The prime site of action of (LH) is the leyding cells where it regulates the testosterone secretion (Guyton, 1987). While, the prime site of action of (FSH) is the Sertoli cells where it stimulates these cells to secrete the (ABP)-Androgen-Binding- Protein and stimulates the mitotic activity of the spermatogonia (Ganog, 1997). However, the secretion of (LH) and (FSH) from the anterior pituitary gland is influenced by a range of factors including: the negative feedback effect of the testosterone hormone as well as, the influences of drugs and chemotherapeutic agents (Poirier *et al.,* 2003).

1.4.8.1.4- Sperm cells:

Spermatozoa (the male gametes) are contained within the semen which is the liquid cellular suspension secreted at ejaculate from the accessory organs of the male reproductive tract (Garner and Hafez, 2000).

Fully formed spermatozoa are elongated cells consisting of an oval, flattened head containing highly compact chromatin comprised of deoxyribonucleic acid (DNA) complexed to a special class of basic proteins known as (sperm protamines) (Clermont, 1972). The anterior end of the sperm nucleus is covered by "the acrosome", a thin, double - layered membranous sac that is layered over the nucleus during the last stages of sperm formation (Eddy, 1988). This cap–like structure, which contains acrosin, hyaluronidase, and other hydrololytic enzymes, is involved in the fertilization process.

While, the tail of the male gamete is composed of the neck, middle, principal, and the end piece which contains the central axoneme responsible for sperm motility (Flechon, 1981). The entire spermatozoon is covered by the plasma membrane (Garner and Hafez, 2000).

1.4.8.1.5- Semen quality and infertility:

Sperm motility is one of the most important criteria of semen quality, because the sperm must be motile to pass through the uterus and Fallopian tubes of female genital tract to achieve fertilization of oocyles in ampulla (Zaneveld, 1978). Macleod and Wang (1979) stated that, the sperm concentration is meaningless unless sperm motility is also taken into account.

However, the ejaculate may be considered abnormal if (< 50%) of spermatozoa showed a decrease in sperm motility, which is also called "asthenozoospermic semen" sample (Lindemann and Kanous, 1989).

Moreover, Colleu and his co-workers (1988) showed that, the spermatozoa from asthenozoospermic samples may be characterized by their defective concentration, motility and morphology, in addition to their relative nuclear immaturity. Therefore, asthenozoospermia is an indication of significant reduction in sperm motility and male infertility. The cyclic adenosine mono-phosphate (cAMP), Calcium and adenosine triphosphatase (ATPase) and energy source play an important role in modulating the functioning of the flagellar axoneme (Lindeman and Kanous, 1989). Liu and

Baker (1992) indicated that, the sperm motility depends on a flagellum, which develops the propulsive force for swimming.

The motility of sperm from proven fertility individuals are faster significantly than values measured for infertile individuals (Shuxiang *et al.*, 1990). Zaneveld (1978) indicated that, the adenosine triphosphate (ATP) was generated by the metabolic events that take place in the cytoplasm and mitochondria of the midpiece. In addition, Amelar *et al.*, (1980) demonstrated that, ATP was necessary for the contraction of flagellum and the concentration of cAMP determined the frequency of the flagellar beating. Moreover, the concentration of cAMP has been shown to have effects on sperm metabolism, motility and fertilization (Aitkin *et al.*, 1986).

On the other hand, Calcium ions (Ca^{+2}) has multiple actions on sperm metabolism, it produces a change in flagellar curvature changing the swimming pattern to the tumbling hyper activated pattern, the second action is antagonistic cAMP and gradually deactivates motility, which influences capacitation, acrosome reaction and gametes fusion (Pilikian *et al.*, 1989 and Tarlatzis *et al.*, 1993). Spermatozoa develop the capacity of motility within epididymis (Hass and Beer, 1989). Hinton and his associates (1979) observed that, the spermatozoa first become motile in the caput of the epididymis, and subsequently acquired the capacity to move in a forward direction in the distal regions of the epididymis. It has been noticed that, the sever asthenospermia and / or sever oligospermia are most often associated with the exposure to chemotherapeutic agents (Francavilla *et al.*, 1990).

A study confirmed that, the spermatozoa with normal morphology swim faster than abnormally shaped sperms (Zaneveld, 1978). Other study showed that, strict morphology to be an important predictor of sub fertility (Berne and Levy, 1988, and Kruger *et al.*, 1986).

It has been suggested that, the increase of abnormalities in the asthenozoospermia is of testicular origin during spermatogenesis spermatozoa from asthenozoospermic samples, may be thus be characterized not only by

their defective concentration, morphology and motility but also by their relative testicular abnormality (Colleu *et al.*, 1988).

Siegel (1993) found that, the reduced percentage of motility with a high percentage of non-viable sperms may predict structural or metabolic abnormalities of sperm that are derived from abnormalities in testicular function or cytological anomalies.

1.4.8.2- Mouse female reproductive system:

The female reproductive organs consist of the ovaries, uterine tubes, uterus, vagina, and mammary glands (Seeley *et al.*, 1996).

1.4.8.2.1- The ovary:

The ovary, unlike the testis, remains in the abdominal cavity, and it performs both an exocrine (egg release) and endocrine function (steroidogenesis) (Beek and Boots, 1974). The predominant tissue of the ovary is the cortex and the primordial germ cells rise extragonadally and migrate through the yolk sac mesentery to the genital ridges (Whithingham and Wood, 1983). The shape and size of the ovary vary both with the species and the stage of the estrous cycle (Hafes, 1987). In the mouse female, ovaries lie ventrally just below the Kidneys within transparent ovarian capsules where a narrow, tunnel – like passage connects the periovarial space with the peritoneal cavity (Hafez, 1987).

1.4.8.2.2- Oogenesis:

The formation, development, and maturation of the female gametes begins early in embryonic life but is not completed until the time of ovulation (Whittingham and Wood, 1983). The primordial germ cells give to a definitive population of oocytes which remains in the prolonged resting (dictyate) stage of the prophase of the first meiotic division until about 12 hr before ovulation (Smith and Engle, 1975).

The fetal ovary can be distinguished from the fetal testis by the thirteenth day of gestation; unlike the arrangement of male germ cells in testicular

cords, the female germ cells are scattered throughout the stroma of the gonad, with some of the somatic cells oriented in rows subdividing the ovary into several compartment (Guyton, 1987). At this time, mitosis ceases, the oogonia undergo a final DNA replication and enter meiosis, and over the next couple of days they pass through loptotene, zygotene and pachytene and finally reach the dictyate stage by 5 days postpartum (Smith and Engle, 1975). Austin and Short (1972) reported a detailed account of histological changed in the ovary from the tenth day of gestation to day 5 postpartum.

Although development of the germ cells in an individual is almost synchronous, the timing of oocyte development varies between strains. The majority of oocytes of CBA mice have reached the dictyate stage at birth (Beek and Boots, 1974).

A close association between the oocyte and somatic cells commences shortly before birth (day 18 of gestation), with the formation of primordial follicles which consist of a single layer of follicular cells surrounding each oocyte.

The initiation of follicular growth from the pool of primordial follicles is continuous throughout the reproductive life of the female. Follicular maturation takes place in (10 – 17) days reach to the postovulatory stage, and it was found that, the follicle growth was sequential so that, those follicles starting to grow continuous until ovulation occurs; or in the absence of sufficient gonadotropin, the follicle becomes atretic (Smith and Engle, 1975).

Remarkably, few female germ cells complete oogenesis and are ovulated. The majority were eliminated by the degenerative process Known as "atresia" which takes place continuously throughout oogenesis and persists until the ovaries were finally depleted of germ cells at the end of the reproductive life of the female (Ganog, 1991).

On the other hand, ovulation, the climax of follicular maturation was found to occur approximately (12 hrs) after the spontaneous surge of the leutinizing hormone (LH) resulting in the follicle rapture and the release of the oocyte

(Whittingham and Wood, 1983). It was found that, ovulation was spontaneous in mice and strains differed in the timing of ovulation (Smith and Engle, 1975).

The number of eggs ovulated in each cycle was approximately constant within a strain and was dependent on the duration of hormonal stimulation rather than an absolute levels of plasma FSH (Ganog, 1997).

1.4.8.2.3- Estrous cycle:

Mouse females were characterized by cyclic events in ovary reflected in the behavioral, physiological and anatomical changes of estrus cycle (Whittingham and Wood, 1983). The estrous cycle is classically consists of four stages: proestrus, estrus, metestrus and diestrus (Hafez, 1987).

Estrus is characterized by sexual receptivity when the female will allow copulation, in the mouse, estrus lasting, to 15 hours is characterized by behavioral changes in the presence of another mouse (Beek and Boots, 1974).

Smith and Engle, (1975) had reported that, vaginal epithelium undergoes well – marked changes during the estrous cycle so that, estrus is characterized by qualifications and cornification of the cells and disappearance of leukocytes. During the 24 hours preceding the next ovulation, epithelial cells become abundant and their size and degree of pyknosis increase toward ovulation (Harris and Naftolin, 1970).

The vaginal smear is thus an excellent indicator of the stage of the estrous cycle. Hafez (1987) declared that, during the active part of the estrous cycle (proestrus and estrus) animals called “in season“. These periods usually occur at intervals of 4 to 18 months, the time and regularity of each interval being and individual and breed characteristics (Gay, 1965). However, table (1.2) shows the characteristics of the sexual cycle in laboratory mice.

Table(1.2): Main characteristics of sexual cycle of laboratory mice according to (Hafez , 1987).

Ovulatory mechanism	Species	Cycle type	Length of cycle (Days)	Duration (hours)	Time of ovulation	Viability of ova (hours)
Spontaneous ovulation	Mouse	Polyestrous (any time)	4 - 5	9 - 20	2 - 3 hrs from estrus onset	10 - 12

1.4.8.2.4- Fertilization and embryo development:

After sperm cells are ejaculated into the vagina, they are transported through the cervix, the body of the uterus to the uterine tubules where fertilization occurs; then the development of the embryo begins after the implantation of the fertilized egg in the uterus (Barlow and Sherman, 1972).
Gardner (1972) had noticed a deviation in the development time of the embryos based on the considerable differences between strain he used. However, Whiltingham and Wood (1983) classified the developmental stage of the mouse embryos from (days 9-19) according to table (1.3).

Table (1.3): Staging of mouse embryos of hybrids between CBA males and C57/ BL/ 6 females.

Gestation Days	Developmental stages	Length (mm)
9	Posterior neurophore, auditory pit	1.2 – 2.5
10	Limb buds. Short tail stump	3.1 – 3.9
11	Long tail	5.0 – 6.0
12	Forefoot and hindfoot plate	7 – 9
13	Anterior footplate indented. Marked pinna	9 – 11
14	Fingers separate distally in forefoot	11 – 12
15	Toes separate	12 – 14
16	Reposition of umbilical hernia, Pinna covering external auditory meatus skin wrinkled, Eyelids closing	14 – 17
17	Fingers and toes joined together	17 – 20
18	Long whiskers	19 – 22
19 – 21	Birth (strain dependent)	23 – 27

1.4.8.2.5- Hormonal control of the estrous cycle:

The physiological and behavioral changes associated with the estrous cycle depend upon a complex integration of pituitary, ovarian and probably adrenal hormones under the integrative control of the hypothalamus (Harris and Naftolin, 1970). The alteration of light and dark controls the timing of events in the cycle (Whittingham and Wood, 1983).

In outline, the ovarian follicles ripen and the oocytes within them mature under the influence of FSH and LH (Beers *et al.*, 1975). Increasing estrogen levels during proestrous induce the spontaneous ovulatory surge of LH (Harris and Naftolin, 1970). After rupture of the follicles estrogen levels fall and corpora lutea are formed but, in the absence of coitus, it remains largely inactive; with estrogen and progesterone levels low, FSH and LH secretion increases and the cycle is repeated (Whittingham and Wood, 1983). The findings of Austin and Short (1972) indicated that follicular fluid contains a nonsreroidal substance which acts together with estrogen to control FSH secretion. However, when considering the précis timing of hormonal events in the mouse's cycle, the animal's environment must be considered, since external influences have a profound effect on the nature of the cycle (Beers *et al.*, 1975).

It was found that, mammalian embryos development required a multitude of sequentially, genetically ordered steps before the embryo was fully formed. Therefore, embryos were prone to intrinsic congenital malfunctions (Jackson, 1974) and were susceptible to interference by various extrinsic agents (Wilson, 1973). Among the nongenetic agents that can adversely alter the development of the mammalian embryo or fetus are potentially scores of chemicals. Laboratory screening experiments have shown that, many chemicals have malignant effects on the unborn (Duplessis, 1972). A drug may produce Its noxious effect either on the fetus, or on the maternally influence extra-embryonic environment; Thus, the various drugs could

perpetrate their teratogenic effects by causing nutritional deficiency, inhibiting enzyme and altering cell membrane function or furthermore by interfering with the mitotic apparatus, nucleic acid metabolism or the DNA methylation (O'Neill, 1998).

1.5- Chromosomes and development:

Mammalian may be exposed to different kinds of extrinsic factors that could affect their normal development through the induction of chromosomal abnormalities (Brach and Hart, 1978). These factors could be physical agents such as (Ultra violet light or X-ray) or they could be numbers of chemical compounds that have the ability to defect the chromosomes (Carano *et al.*, 1979). One of the most important chromosomal abnormalities is the structural abnormalities called (sister chromatide exchange–SCE) which is known to be a manifestation of DNA damage and misrepair leading to the damage of the chromosomes (Latt *et al.*, 1981). Sister Chromatide exchange abnormalities is seemingly related to mutagenesis (Sasaki, 1982), morphological transformation induction in mammalian cells by various, physical and chemical carcinogens (Popescu and Diapolo, 1982), and the teratogenecity of many chemical agents (Karm, 1982).

In recent years, more attention has been devoted to study the relation of sister chromatide exchange occurrence with health and disease (Rabbits, 1994). Shubber and his partners (1991) had confirmed in their study that, BALB/c mice showed spontaneous frequencies of sister chromatide exchange and chromosomal aberrations in bone marrow cells which was increased due to the effect of a number of carcinogens, which causes cryptogenic damage.

Other studies made also by Shubber *et al.,* (1985) and Shubber *et al.,* (1999), showed that, sister chromatide exchange and chromosomal aberration could resulted from the effect of DNA damaging agents which caused DNA strand breakage in the treated mice leading to the abnormalities (Shubber and Salih, 1988). On the other hand, chromosomal abnormalities in tumors were recognized at the end of the twentieth century, but their significance has only

recently became clear (Mange and Mange, 1999) and it was found that, cancer might result from somatically acquired genetic changes; sometimes associated with inherited predisposing mutations (Rabbits, 1994). There are many cytogenetic changes that may lead to cancer development including: deletions, translocations and inversions (Mange and Mange, 1999).

Others have reported consistent defects in dozens of different cancers. These defects included: translocations associated with leukemia and lymphoma, deletions or missing pieces, which occur in solid tumors such as lung and kidney cancers and extra – chromosomes which were linked to various other tumors (Gold, 1983). On the other hand, nutrition researches had highlighted the role of several nutrients in regulating the genome machinery (Frisco and Choi, 2002). A number of vitamins and micronutrients are substrates and /or repair and the expression of genes (Fenech and Ferguson, 2001). It has been documented that, the deficiency of such nutrients may result in the disruption of genomic integrity and alteration of DNA methylation; Thus, linking nutrition with modulation of gene expression (Robertson and Wolffe, 2000).

The discovery of polymorphic enzymes involved in critical steps of nucleic acids metabolic pathways together with the chemotherapeutic agents had contributed insights to the production of abnormal chromosomes and the expression of defective phenotypes (Frisco and Choi, 2002).

1.6- Major techniques to study DNA methylation:

The techniques used to analyze DNA methylation can be divided into two categories: one of which leads to sequence - unspecific results (sequence – unspecific methods), and the second technique allows a precise location of some or all of the methylated sites within a given DNA target sequence (sequence- specific methods) (Jost and Saluz, 1993).

The first category of the analysis techniques could be used to analyze different modified bases and to quantify them in an organism, organ or tissue, but it doesn’t provide any information about the precise location of the

modified site within the given genome (Sano *et al.*, 1980), into this category fall the immunological (Achwal *et al.*, 1984), the chromatographic (Wagner and Capesius, 1981), the electrophoresis (Kuo *et al*., 1980), and the enzymatic hydrolysis of the target DNA (Ford *et al*., 1980). Another sequence – unspecific approach involves the use of methylation – sensitive restriction endonucleases where different digested genomic DNA's were compared upon separation by gel electrophoresis (Adams and Burdon, 1985).

However, methylation sensitive restriction enzymes are often used for a comparison of the resulting DNA restriction fragments by Southern blot analysis (Bird and Southern, 1978), which allows the analysis of about 10% of all potentially methylated sites of a given genome. This procedure has, however, certain draw- backs in that, hemimethylated DNA's often remain undetected (Gruenbaum *et al*., 1981). Some investigators had applied (RAPD-PCR) technique to study DNA methylation (Jost and Saluz, 1993). This technique was based upon the fact that, a short oligonucleotide or randomly chosen sequence (primer) of usually 10- bases, when mixed with genomic DNA (template) and a thermostable DNA polymerase (Taq polymerase) and subjected to the temperature cycling conditions of PCR will allow the amplification of several DNA fragments (Innis *et al*., 1990; Dennis – Lo, 1998). Singer – Sam *et al*., (1990) had applied this technique for methylation studies by cleaving the total DNA to completion with methylation – sensitive restriction endonucleases for example HpaII, and during the RAPD-PCR reaction, primers will bind randomly to the DNA fragments allowing the exponential amplification products were then separated on agarose gels and detected by ethidium bromide staining (Jost and Saluz, 1993).

Furthermore, new techniques were recently developed which circumvented chemical cleavage reactions, based on bisulfate – induced modification of the genomic DNA under conditions where cytosine was converted to uracil and the 5- methylcytosine remained unchanged (Jost and Saluz, 1993).

The target sequence was then exponentially amplified using PCR reaction and strand - specific primers. Upon sequencing of the amplified DNA, all uracil and thymine residues become detectable as thymine and only 5-methylcytosines as cytosine; by a comparison of the modified sequence with the unmodified one it became possible to analyze the methylated cytosines.

Another interesting approach was also developed to study DNA methylation involved in the use of polyacrylamide gel electrophoresis for the analysis of the cytoplasmic proteins of the brain, liver, kidney and in some cases spleen and testis of rat and mouse (Comings and Tack, 1972). On the other hand, the detergent (sodium dodecyl sulphate, SDS) has long been recognized as a powerful protein denaturant and solubilizing agent, which induces conformational changes in proteins at low concentration (Steinhardt and Reynolds, 1969). Several workers had found that, at SDS concentrations higher than (1.4 g) of detergent would bind to (1g) of protein, resulting in a complex with rod-like shape, the length of which varies uniquely with the polypeptide molecular weight (Pitt- Rivers and Impiombato,1968; Reynolds and Tamford, 1970). From the non- specific nature of the binding of SDS to a wide variety of proteins, representing many different structural types, it can be inferred that, complex formation requires conformational changes of similar character, with each complex formation requires conformational changes of similar character, with each complex having a uniform type of structure (Westermeier *et al.*, 2001). SDS binds readily to proteins in constant ratio, producing complexes with constant charge per unit mass (Shapiro *et al.*, 1967). This fact explains the findings of Weber and Osborn (1969), in which they had reported that, proteins could be dissolved in high concentrations of SDS exhibited electrophoresis motilities in polyacrylamide gels which were a direct function of their sub- unit molecular weights. The technique of SDS – PAGE, has been rapidly grown in popularity and an economic method for determining protein sub-unit molecular weights and it requires only microgram quantities of protein (Westermeier *et al.*, 2001).

Chapter Three

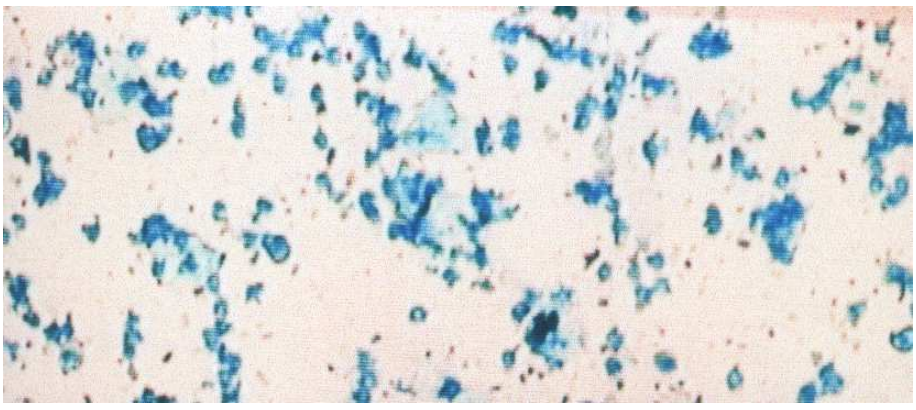
Results

3.1- Establishment of fibroblast cell culture:

3.1.1- Detection of the estrous cycle and the establishment of mating colonies:

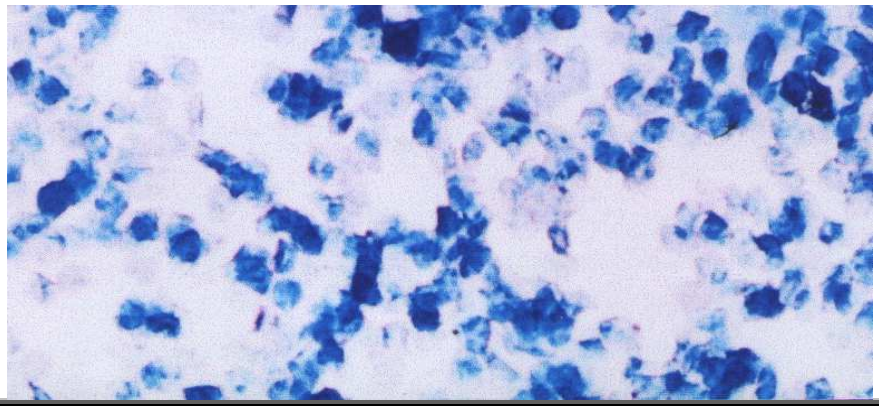
The onset of puberty in the mouse female occurs from about four weeks onward after birth (Whitingham and Wood, 1983).

In this regard, about thirty females of ***Swiss albino*** strain were selected in the age of eight weeks old and attempts were made to detect their estrous cycle - events where results of repeated vaginal smears divided the estrous cycle of the experimental females into a sequence of four phases as shown in figures (3.1a, b, c, &d).

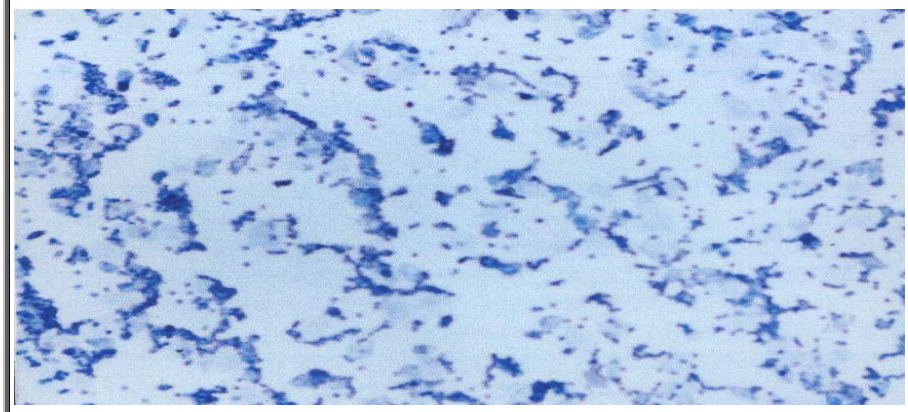


a- Proestrus phase

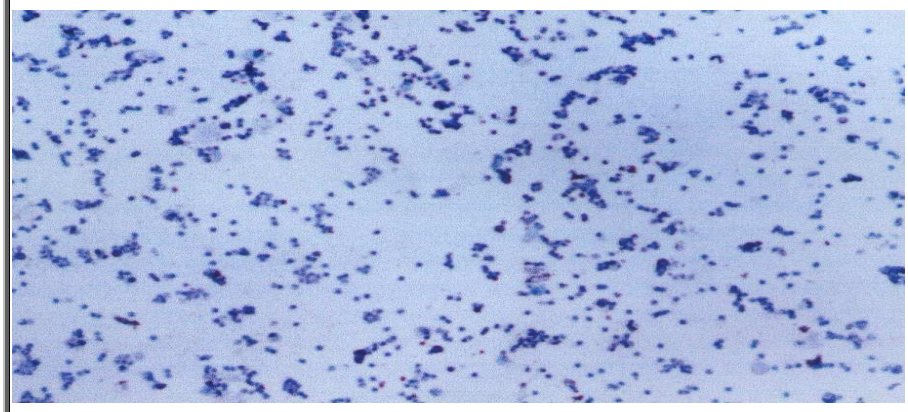
Figure (3-1): The sequence of mouse female estrous cycle: a-Proestrus, b-Estrus, c-Metestrus and d- Diestrus phases. Slides were stained with (0.05%) methylene blue and examined under (40x) objective of a light microscope.



b-Estrus phase



c- Metestrus phase



d- Diestrus phase

Therefore, the viability maintenance of the culture required a periodic medium change every (three to five) days of incubation followed by a subculture at the ninth day of incubation.

Table (3.1) summarize various feature of the initiated fibroblast – like cell culture obtained through this work, which was given the name of "fibroblast-like AME11D1/2cell culture" where, AME11D1/2 was the abbreviation of (Asal ,Mouse Embryo, Dissected at the age of 11 .5 days of gestation).

Table (3.1): The profile of the fibroblast like AME 11 D1/2 cell culture

Animal	Mouse
Scientific name	***Mus musculus***
Strain	***Swiss albino***
Tissue	Embryonic
Age	11.5 days old
Disagregation method	Primary explants techniques
Selective medium	RPMI 1640 + 10%FCS
Passage method	Trypsinization
Morphology	Fibroblast like cell
Life span	Finite, 6-7 passages
Classification	Normal non transformed cells
Established by	Asal Aziz

3.1.3- Effect of different media or the seeding efficiency and cell morphology of mouse fibroblast-like and plasmacytoma SU99 cell cultures:

Two types of cell cultures were used to certain the effect of three types of media on the growth of two mouse cells cultures, a normal non – transformed fibroblast – like and a transformed culture of plasmacytoma SU99 cells. Measurements of seeding efficiency, typical and atypical cell morphology, were considered as parameters for the assessment of the biological performance of the used media. Three types of media were used (medium RPMI 1640, Eagle's minimal essential medium and medium 199).Figure (3-5) demonstrated the difference in the seeding efficiencies.

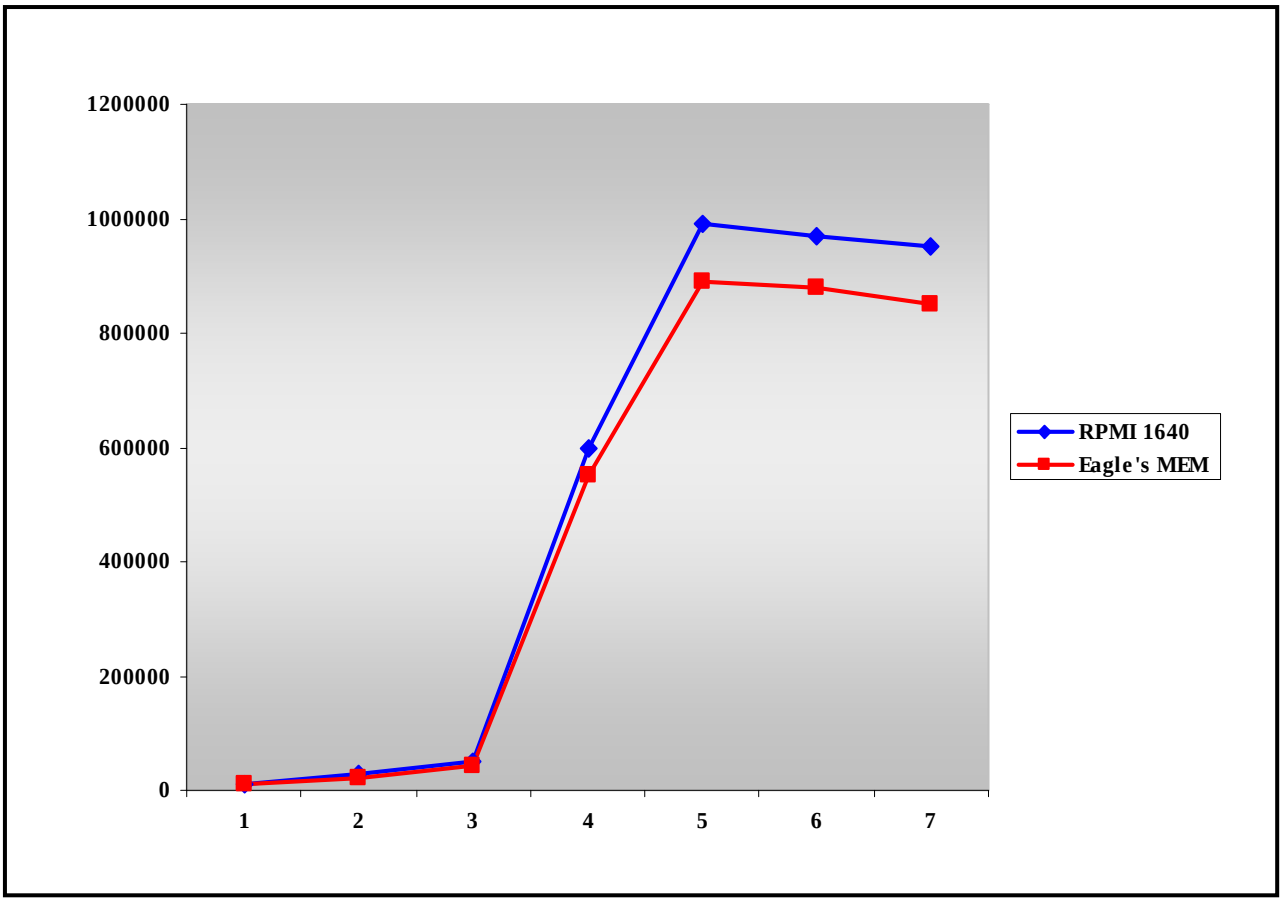


Figure (3.5): Growth of fibroblast- cells on RPMI 1640 and Eagles minimal essential medium.

The observed seeding efficiency of RPMI 1640 medium was (98%) While, the seeding efficiency that was calculated for cells grown on Eagle's minimal essential medium for seven days according to the same equation was (85%). Thus, the difference in the seeding efficiency between the two types of media used in this study was (10%). This difference in the seeding efficiency might indicate that, RPMI 1640 medium was more efficient in supporting the growth of fibroblast-like cell cultures, in comparison with the results obtained from cells grown on Eagle s minimal essential medium.

On the other hand, the growth of plasmacytoma SU99 cell cultures in RPMI 1640 medium or Eagle's minimal essential medium demonstrated lower difference between the seeding efficiencies calculated. The obtained results showed that both RPMI 1640 medium and Eagle's minimal essential medium had almost the same efficiency in supporting the growth of plasmacytoma SU99 cells. Calculated seeding efficiency for plasmacytoma SU99 cells grown on RPMI 1640 medium was (98%), and the seeding efficiency of plasmacytoma SU99 cells cultivated was (95%).

Accordingly, the obtained results concerning the values of seeding efficiencies of both media showed a slight difference of (3%) only.

Thus, it was possible to conclude that both media had showed almost same biological performance in the assessment of plasmacytoma SU99 cells

growth. Figure (3-6) showed the effect of both media on the growth of plasmacytoma SU99 cells.

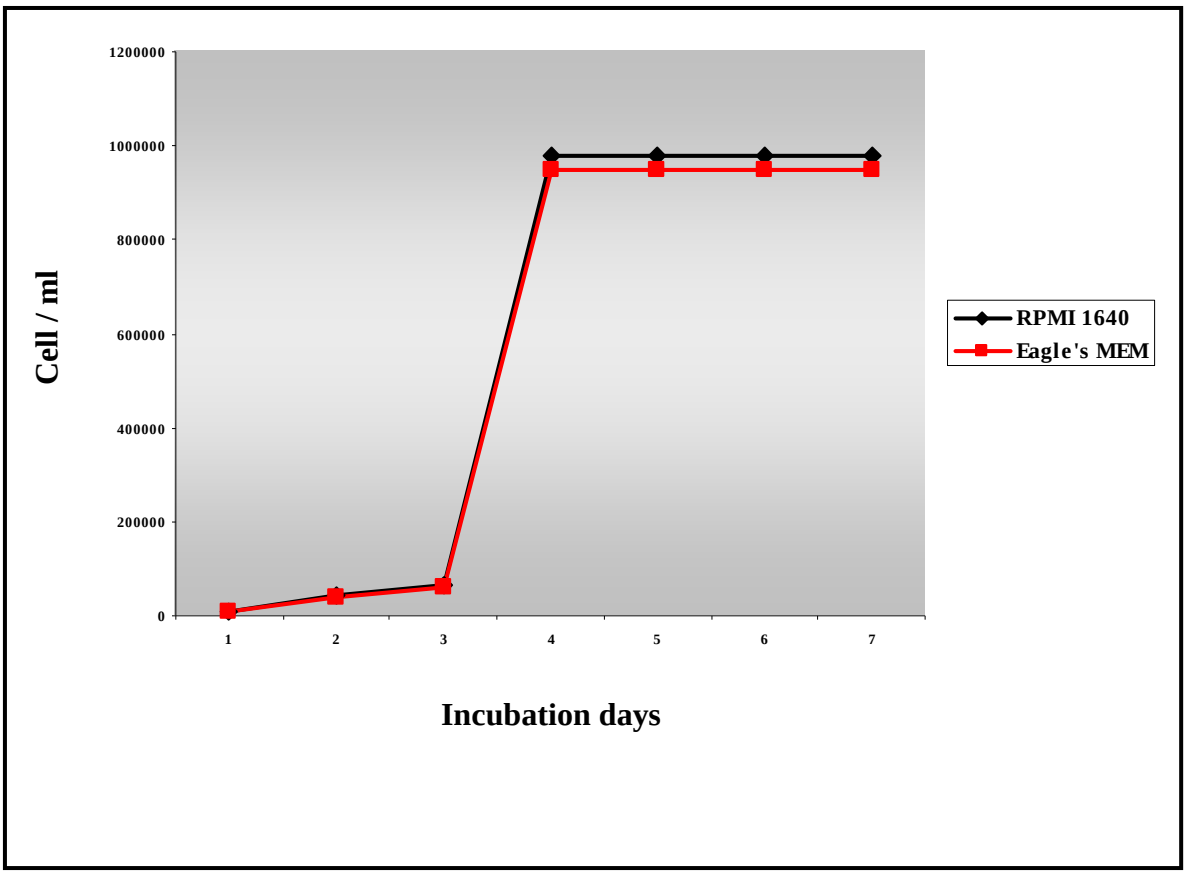


Figure (3-6): Growth of plasmacytoma SU99 cells on RPMI 1640 and Eagle's minimal essential medium.

On the other hand, medium 199 did not support the growth of either type of cells. This was indicated by the absence of cell adherence to the substrate of the tissue culture flasks.

Morphological examination of the fibroblast - like cell cultures grown on RPMI 1640 medium or Eagle's minimal essential medium showed features of typical cell morphology of both cell types.

Figure (3-7) showed the typical cell morphology of Plasmacytoma SU99 cells grown on RPMI 1640 medium.

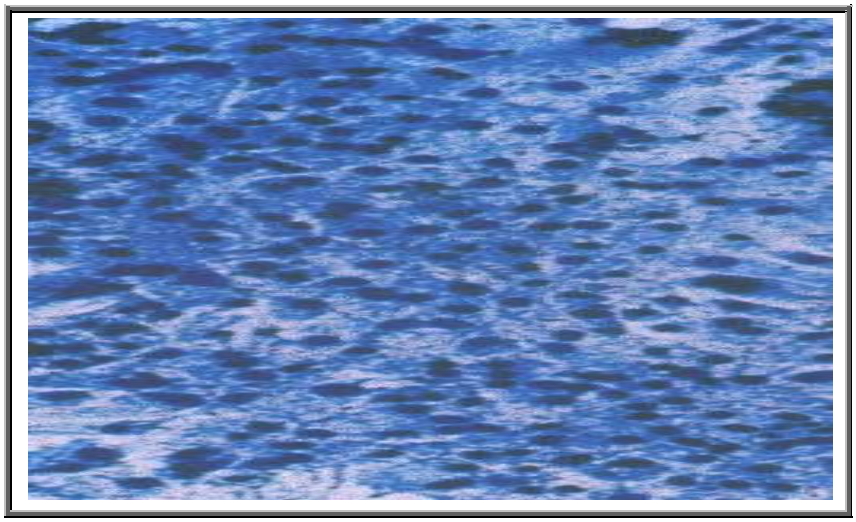


Figure (3-7): Morphology of plasmacytoma SU99 cell cultures grown on RPMI 1640 medium for seven days. Cells were examined at (40x) objective of an inverted microscope and were stained with Giemsa stain.

The careful examination of plasmacytoma SU99 cells of this figure showed adherent fibroblastic, bipolar, short cells with different sizes.

While, some other cells appeared multipolar, with single centric nuclei, while other cells were binucleated.

At higher densities, plasmacytoma cells showed a pattern of a multilayer growth and cells were independent of contact inhibition.

3.1.4- In vitro effect of the treatment with 5-azacytidine on the fibroblast–like AME11D1/2 and the plasmacytoma SU99 cell cultures:

3.1.4.1- Cytotoxicity measurements:

3.1.4.1.1- Short term- viability assay

Ninety-six well tissue culture plates were used to ensure the toxic effect of 5-azacytidine on both types of cells where a cytotoxic effect was reflected by different concentrations of 5-azacytidine on the viability of the cultivated cells as table (3.2) shows.

Table (3.2): Cytotoxic effect of different concentrations of 5-azacytidine on fibroblast like and plasmacytoma SU99 cells.

Azacytidine concentration (µg/µl)	Optical density of fibroblast cells	Optical density of plasmacytoma cells
250	0.20	o.235
125	0.22	o.25
62	0.24	0.27
31	0.26	0.29
15	0.276	0.30
7	0.29	0.31
3	0.30	0.325
1	0.32	0.34
0.9	0.34	0.36
0.5	0.36	0.38

3.1.4.1.2- Long term- Survival assay:

Cultures of fibroblast – like and plasmacytoma SU99 cells were seeded in a density of (100 cell/ ml) and were treated with different concentrations of (0, 1, 3, 5 and 7 µM) of 5-azacytidine where cell inhibition was determined by observing the decrease in the values of plating efficiency which were fixed with methanol and stained with Giemsa stain after seven days of incubation.

The plating efficiency was determined by scoring the number of single cells counted under the inverted microscope, and results were expressed as a percentage of cells plated as it was demonstrated in table (3.3).

Table (3.3): Cytotoxic effect of different concentrations of 5-azacytidine on the plating efficiency of the fibroblast- like and the plasmacytoma SU99 cells. Cultures were seeded at a density of (100 cell / ml) and were incubated at 37ºC and 5% CO_2 for seven days.

Concentrations of 5-azacytidine (µM)	Plating efficiency %	
	Fibroblast like cells	Plasmacytoma SU99 cells
0	100	100
1	45	65
3	21	37
5	3	16
7	0	5

It could be noticed that, different concentrations of 5-azacytidine had toxic effect on both types of cells, where by the fibroblast-like cells were more sensitive to the toxic effect of 5-azacytidine than the plasmacytoma SU99 cells. This was evidenced by the decrease in the number of viable-death percentages of fibroblast-like cells cultures at various concentrations of 5-azacytidine.

3.1.4.2- In vitro effect of 5-azacytidine on the growth characteristics of the fibroblast-like and the plasmacytoma SU 99 cell cultures:

One of the interesting effects observed on fibroblast-like cells after treatment with concentrations of (0,1.3,5,7&9µM)of 5-azacytidine beside the cytotoxic effect of the drug on the cells , was an effect on their growth characteristics represented by the loss of the adherering ability of the treated cells. In other words, those cells which survived the treatment couldn't produce monolayer and cells remained single without contacts with each other as shown in figure (3.8).

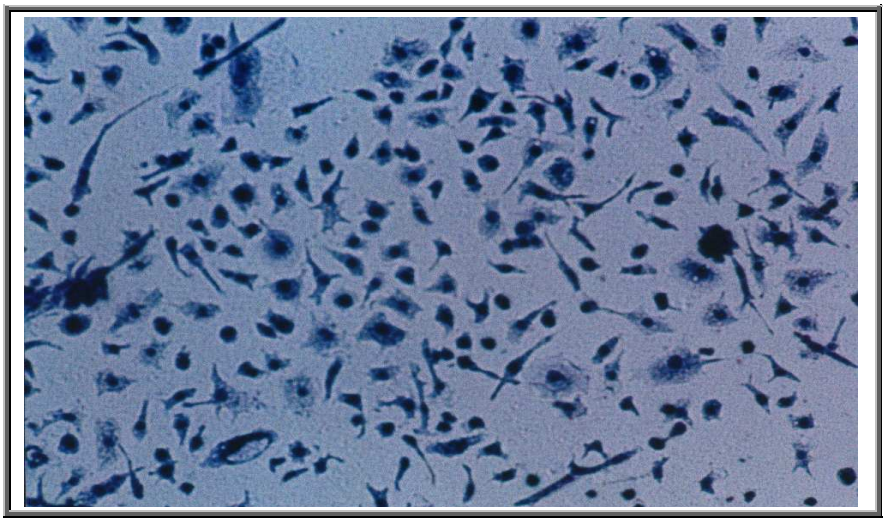


Figure (3.8): Morphology of fibroblast – like cells cultivated on RPMI 1640 medium for two weeks after their treatment with (1 µM) of 5-azacytidine for 24 hours. Cells were stained with Giemsa and examined under (40x) objective of an inverted microscope.

This figure showed cells with the following morphological characteristics:

1- Cells grown after treatment appeared single.
2- Various shapes of cells were observed; they ranged between long bipolar fibroblastic, short bipolar and rounded cells.

3- Cells were different in their length which ranged between (2.5-0.2 µm) as compared with the untreated cells length that ranged between (3-8 µm).
4- No monolayers were observed after two weeks of cultivation on RPMI 1640 medium.

On the other hand, Plasmacytoma SU99 cells treated with (0, 1, 3, 5, 7, and 9 µM) concentrations of 5-azacytidine produced subcultures with the following characteristics:

1- Cultures appeared as single cells.
2- Cells were almost equal in their size which was about (4 µm), shaped as short bipolar fibroblastic with an eccentric nuclei as shown in figure (3-9).
3- No monolayers were observed after two weeks of cultivation on HAT-medium.

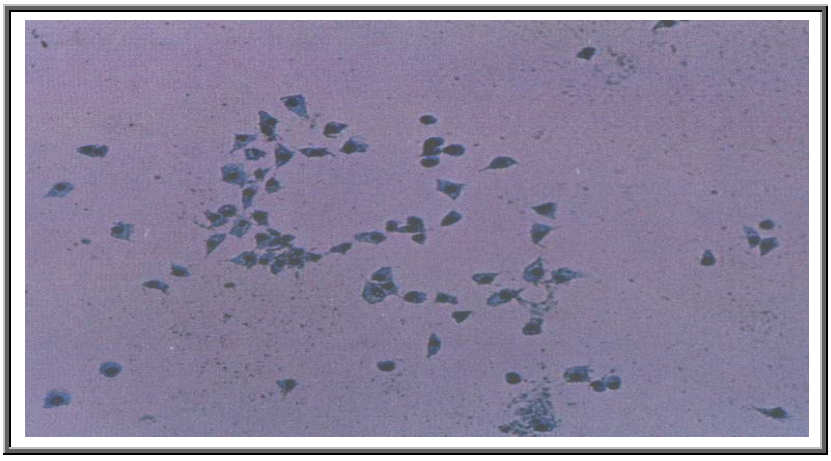


Figure (3-9): Morphology of plasmacytoma SU99 cells on HAT-medium after treatment with (3 µM) of 5- azacytidine cells were stained with Giemsa and examined under (40x) objective of an inverted microscope.

However, Treatment of the plasmacytoma SU99 cells deficient in hypoxanthine-guanine-phosphoribosyltransferase(HPRT)with 5-azacytidine

produced cultures that were (HPRT- positive) and cells were able to grow on HAT- medium.The average number of HPRT- positive cells observed was dependent on the concentration of 5-azacytidine used as table (3.4) shows:

Table (3.4): Average of HPRT-positive plasmacytoma cells produced after treatment with (0, 1, 3, 5, 7&9 µM) of 5-azacytidine.

Azacytidine concentration(µM)	Average of reverted cells %
0	0
1	60
3	87
75	45
7	28
9	5

It was possible to notice two important phenomena from the careful examination of the results of this table: first was the (Tumor cell reversion process) where a number of plasmacytoma cells had reverted to the wild type phenotype after their exposure to different concentrations of 5-azacytidine. On the other hand, a second important phenomenon was also detected (The hermetic effect), where the concentration of (3µM) of 5-azacytidine scored the higher rates of reverted cells through the hypomethylating activity of this agent when it was incorporated into the DNA and prevented the subsequent interaction of DNA with DNMTase that in turn resulted in the activation of the (HPRT-genes).While the higher concentrations of 5-azacytidine brought up cytotoxic effects rather than inducing higher rates of reversion due to the hermetic effect so that, cells reached probably to a degree of saturation that any increase in the 5-azacytidine concentrations will be cytotoxic rather than hypomethylating.

3.2- In vivo effects of 5-azacytidine and folic acid on white mice:

Healthy, adult mice males and females were assigned to treatments with 5-azacytidine or folic acid, three times a week for six weeks. Animals were examined every morning during the time course of the treatments for signs of toxicity such as: hair fall, laziness, faintness and hemorrhage.

It was found experimentally that, all males and females had survived the six weeks treatments of (8mg/kgbody weight) of 5-azacytidine or (0.1 mg/kg body weight) of folic acid.

Table (3.5): Dose and duration time course of animals' treatment with 5-azacytidine and folic acid.

Treatment	Dose/treatment	Duration	Animal/group
5-azacytidine	8mg/kg	18treatments/6weeks	7
Folic acid	0.1mg/kg	18treatments/6weeks	7

3.2.1- In vivo Effects of 5-azacytidine and folic acid treatments on mice body weight:

The results in tables(3.6)and(3.7) indicates that, treatment with (8mg/kg body weight) of 5-azacytidine had resulted in a highly significant decrease ($p < 0.001$) in the final body weight values of both treated mice males and females animals compared with the body weight values of the control group. While, treatment with folic acid revealed a non-significant increase ($p>0.05$) in the final body weights.

Table (3.6): Effects of 5-azacytidine on mice body weight.

Azacytidine concentrations	Treated males body weight/week	Treated females body weight/week	Control males body weight/week	Control females body weight/week
0	**24**	**23.55**	**24**	**23.5**
0.6	**24**	**23.35**	**24**	**23.5**
1.2	**23.9**	**23.22**	**24**	**23.5**
1.8	**23.7**	**23.14**	**24**	**23.5**
2.4	**23.5**	**23.10**	**24**	**23.5**
3.0	**23.2**	**22.98**	**24**	**23.5**
3.6	**22.9**	**22.95**	**24**	**23.5**

It could be noticed from this figure that, treated males with 5-azacytidine had lost about (95.41%) of their body weights at the end of the treatment course compared to the body weight values of the control group .While, mice females had lost about (97.65%) of their body weights at the end of the treatment course. Thus, it was suggested that, mice females were more sensitive to the cytotoxic effects exerted by the 5-azacytidine on mice body weight than males. While figure (3.10) shows no effect of folic acid administration on mice body weight.

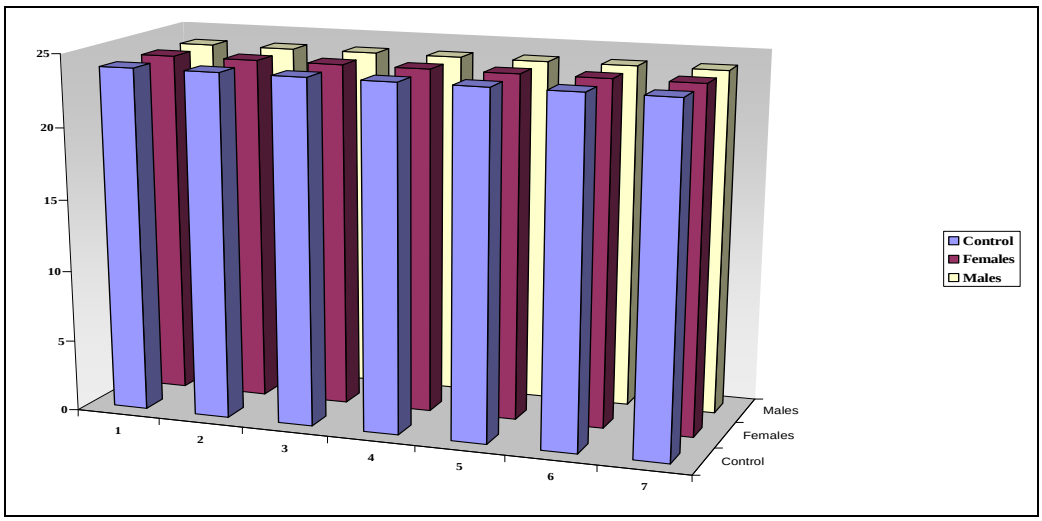
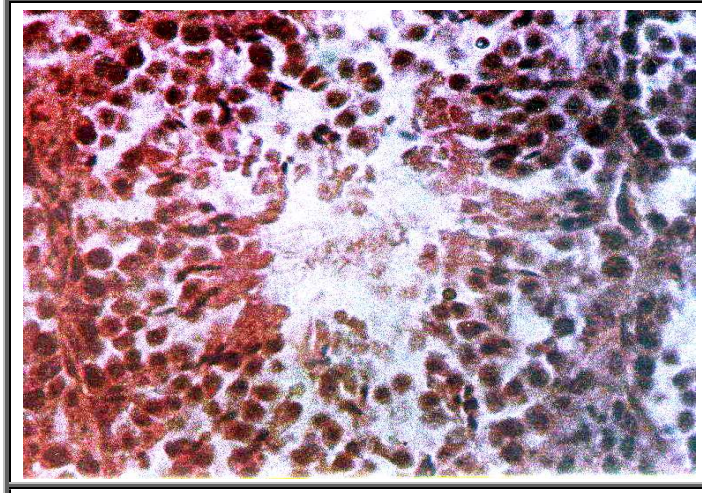


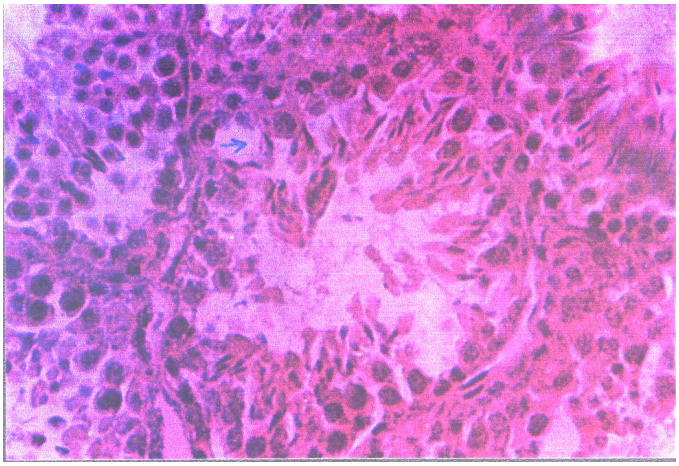
Figure (3.10): Effects of folic acid administration on mice body weight.

3.2.2- In vivo effects of 5-azacytidine and folic acid treatments on mice male reproductive organs:

It was suggested through this work that, a number of germ cell abnormalities were detected in a series of testicular cross-sections could be the result of the treatment for six weeks of mice males with (8mg/kg body weight) of 5-azacytidine. The possible effects were shown in figure (3.11).

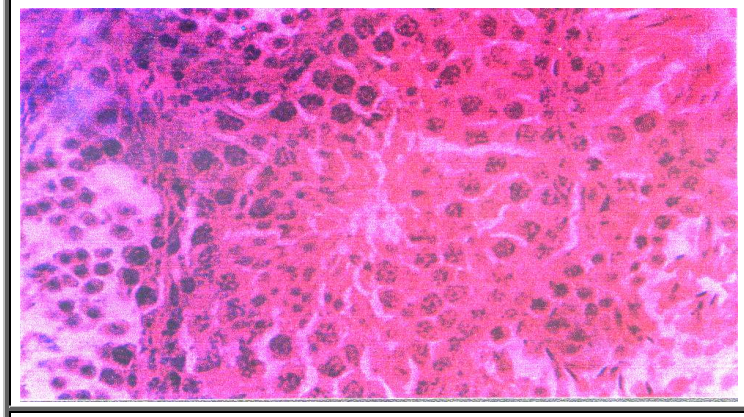


a- Normal seminiferous tubule.

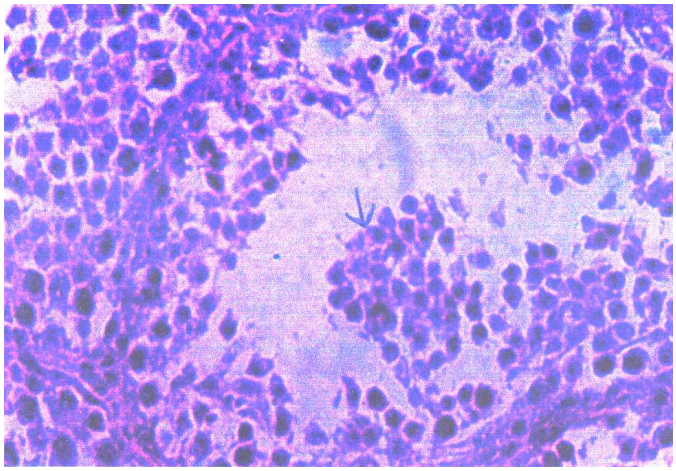


b- Abnormal seminiferous tubule with vacuoles.

c- Abnormal wide seminiferous tubule with giant cell formation (arrow).



d- Degeneration of the seminiferous tubule.



Sloughing of the immature germ cells into the seminiferous lumen (arrow).

Figure (3.11): Effects of 5-azacytidine on the testes histology of mice males. Paraffin-embedded cross sections of both right and left testes stained with hematoxylin and eosin, viewed under the (100x) objective of a light microscope.

A detailed examination of this figure showed a number of abnormalities which was included: the formation of vacuoles in figure (3.11b), formation of multinucleated giant cells with wide tubule in figure (3.11c), degeneration of the tubule in figure (3.11d) and sloughing of immature germ cells into the lumen in figure (3.11e).Therefore, treatment of males with 5-azacytidine had caused a disorganization of the germ cells in the seminiferous tubule.

In addition, a deleterious effect of 5-azacytidine administration was also recognized on the major features of the epididymal sperms and results were shown in table (3.7).

Table (3.7): *In vivo* effects of (8mg/kg body weight) of 5-azacytidine administration for six weeks on the epididymal sperms of the treated mice males.

Sperm characteristics	Control group	5-azacytidine treated group
Motility	Excellent (95%)	Good (70%)
Concentration (sperm /ml)	$6*10^6$	$4*10^6$
Viability	95%	75%
Morphological abnormalities	10%	50%

Evaluation of the results shown in this table had demonstrated that, treatment of mice males with 5-azacytidine produced various effects on the epididymal sperms including the major features of (motility, concentration, viability and sperm morphology).

Determination of the percentage of motile sperm cells in the semen specimen was critical in the evaluation of the semen quality. The results obtained after the intraperitonial injection of (8mg/kg body weight) of

5-azacytidine for six weeks had revealed a decrease of (25%) in sperm motility when compared with the control group.

Also, the reported results showed a decrease of (200.000 sperm/ml) in the sperm concentration of the 5-azacytidine treated group, when compared with the sperm concentration of the control group. Figure (3.12) shows the *in vivo* effect of 5-azacytidine on sperm concentration.

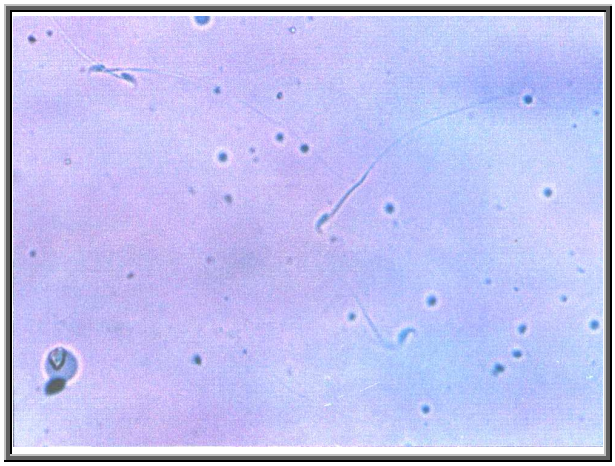
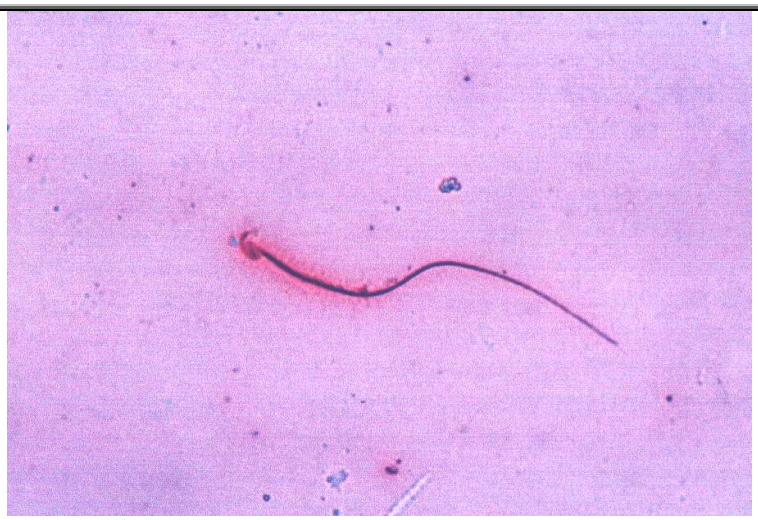


Figure (3.12): *In vivo* effect of (8mg/kg body weight) of 5-azacytidine treatment for six weeks on the testes histology of mice males. Paraffin-embedded cross sections of both right and left testes mounted on slides, stained with hematoxylin and eosin, viewed under the (100x) objective of a light microscope.

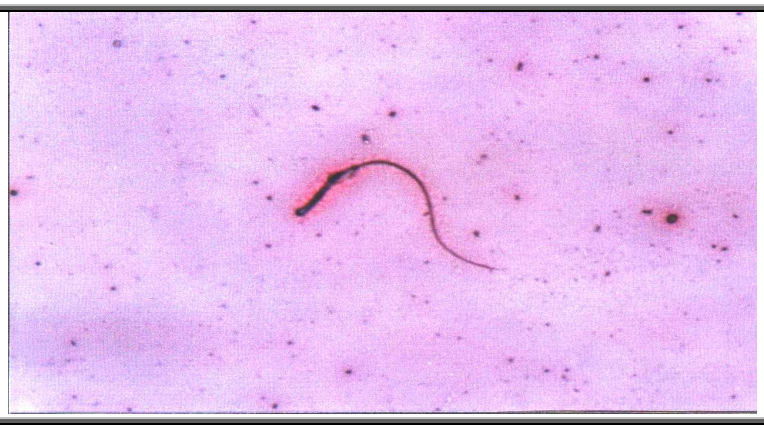
Moreover, this study revealed that, there was an increase in the percentage of dead (red colored sperm head due to eosin stain) after treatment with 5-azacytidine when compared with the control group.

Morphological analysis of sperms was an important aspect in the assessment of sperm function. The obtained results showed an increase in the percentage of morphologically abnormal sperms after treatment with 5-azacytidine.Morphological abnormalities was shown in figure (3.13b-i).

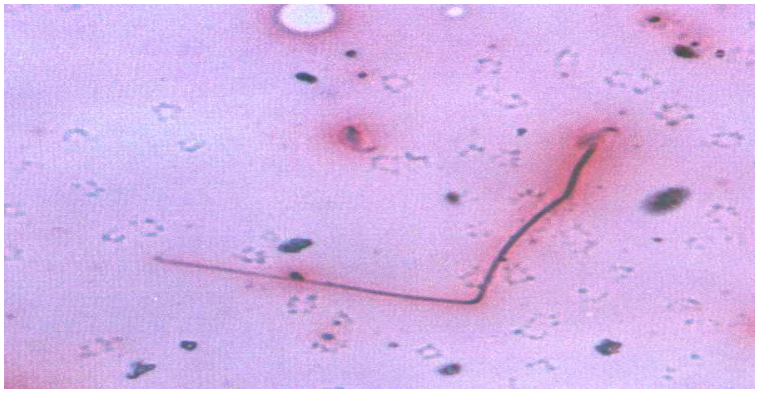


a- Normal sperm morphology of about (7mm) in length.

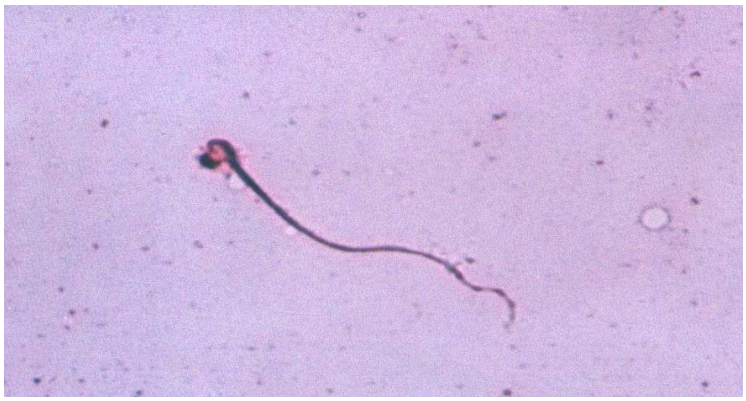
Figure (3.13): Effects of 5-azacytidie on sperm morphology.



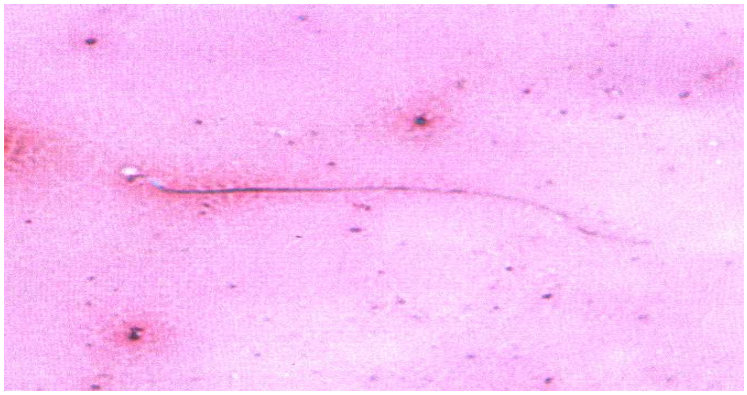
b- Abnormal bent sperm head.



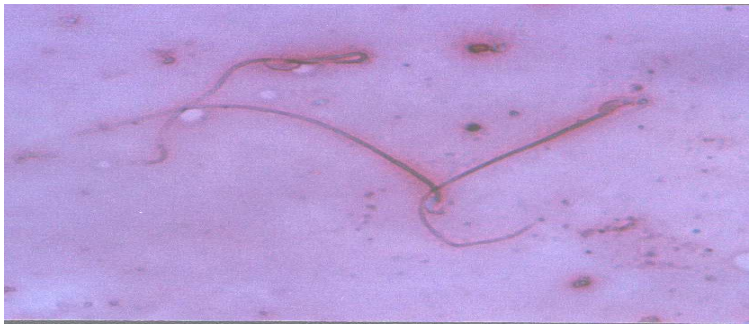
c- Abnormal hammer sperm head.



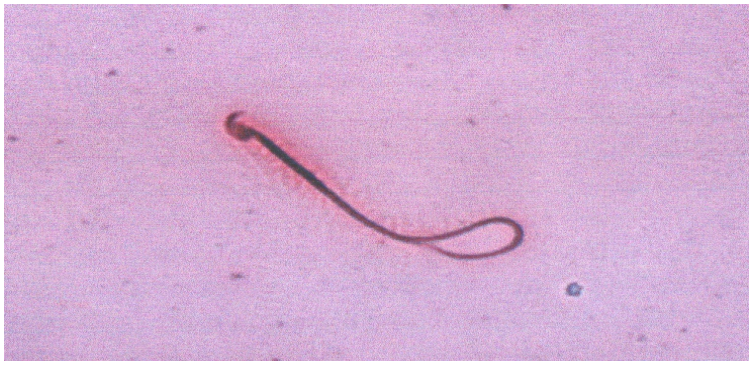
d-Vacuolated sperm head with short sperm tail (5mm) in length.



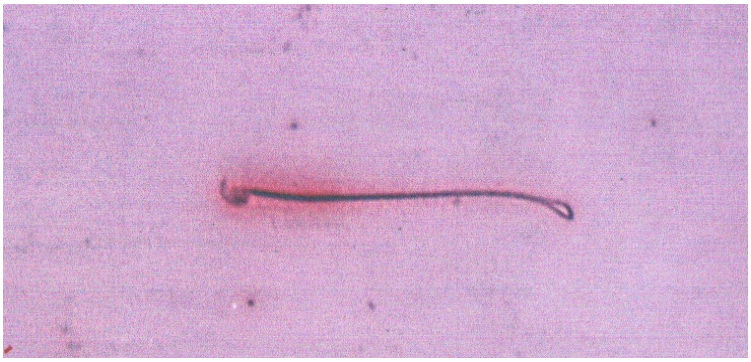
e- Abnormal microcephaly tapered sperm head.



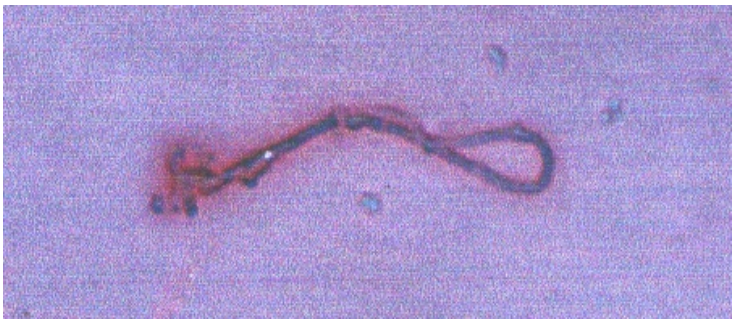
f- Abnormal adherent head, microcephaly tapered (right) and abnormal tail, dancing cup (left).



g- Abnormal coiled tail piece.



h- Abnormal short tail (4mm) in length.



i- Acephaly with cytoplasmic extrusions and coiled tail.

Figure (3.13): *In vivo* effect of (8mg/kg body Weight) of 5-azacytidine on sperm morphology in mice. Specimens stained with eosin-nigrosin stain, examined under (40x) objective of a light microscope.

Results of table (3.8) on the other hand, represented the percentage of the epididymal sperm abnormalities observed after mice male exposure to 5-azacytidine.

Table (3.8): *In vivo* effect of (8mg/kg body weight) of 5-azacytidine on the morphology of the epididymal sperms of treated mice males in (200 slides) stained with eosin-nigrosin stain and examined under (40x) objective of a light microscope.

Sperm morphological abnormalities	Frequency in 100 slides	
	5-azacytidine Treated group	Control group
Acephaly	1	1
Microcephaly	1	–
Adherent head	10	4
Bent head	5	–
Hammer head	3	–
Tapered head	5	–
Vacuolated head	1	–
Total head defects	26%	5%
Coiled tail	17	3
Short tail	7	2
Tail defects	24%	5%
Total defects	50%	10%

On the other hand, the data obtained from figure (3.14), that represented the results of the histological examinations of testes dissected- out from animals treated with (0.1 mg/kg body weight) of folic acid had revealed that, the organization of the germ cells in the seminiferous tubules examined was not affected by the treatment of the mice males with this vitamin.

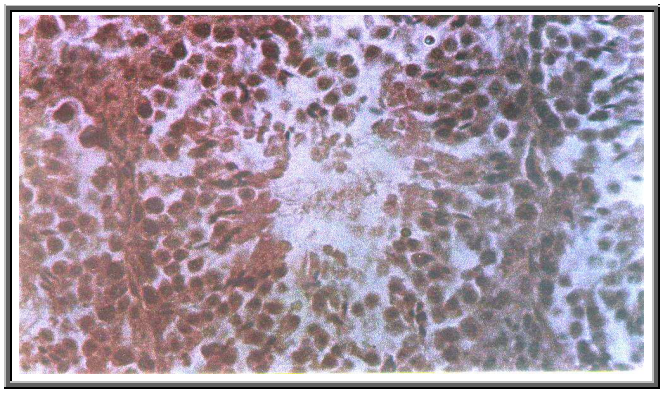


Figure (3.14): *In vivo* effect of (0.1mg/kg body weight) folic acid on testes histology .Paraffin –embedded cross-sections stained with hemotoxylin and eosin .Examinations were made under (40x) objective of a light microscope.

Moreover, epididymal sperms were also been subjected to investigation under the light microscope in order to evaluate the effect of folic acid on sperm's major functions and table(3.9) shows the results of these examinations.

Table (3.9): *In vivo* effect of (0.1mg/kg body weight) of folic acid administration for six weeks on the epididiymal sperms of the treated mice males.

Sperm characteristics	Control group	Folic acid treated group
Motility	Excellent (95%)	Excellent (95%)
Concentration (sperm /ml)	$6*10^6$	$6.5*10^6$
Viability	95%	95%
Morphological abnormalities	10%	10%

However, the careful examination of this table had possibly indicated that, treatment with folic acid had no effect on the major features of the epididymal sperms. In addition, results of figure (3.15) showed that the concentration of sperms was also not affected by the treatment.

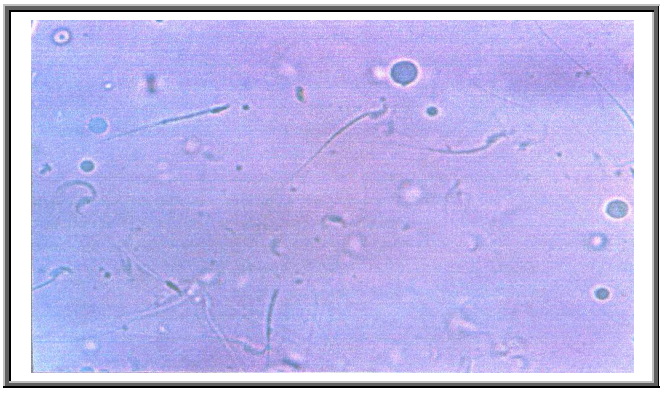


Figure (3.15): *In vivo* effect of (0.1 mg/kg body weight)of folic acid on the numbers of sperms in a microscopic field of unstained specimen, examined under (40x) objective of a light microscope.

3.2.3- In vivo effects of 5-azacytidine and folic acid treatments on mice male fertility rate:

The effects of the *in vivo* treatments of mice males with 5-azacytidine or folic acid on their fertility was assessed by determining the ability of males to mate with females scored by detecting the plugs in female's vagina for the next two mornings. The data were shown in figure (3.16).

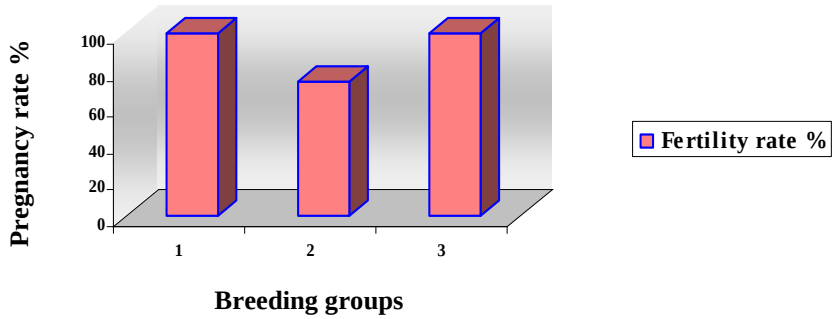


Figure (3.16): *In vivo* effects of 5-azacytidine and folic acid on the fertility rate of the treated males reflected by the percentage of pregnant females. Where (1) = control, (2)= 5-azacytidine, (3)= folic acid.

Results of this figure showed a reduction of (23.7%) in the fertility rate of the treated mice males with (8 mg/kg body weight) of 5-azacytidine, where only (22 mice females) were detected to be pregnant within a breeding group consisted of (30 mice females mated with 20 mice males treated with 5-azacytidine) indicating that, paternal exposure to 5-azacytidine could possibly affect the fertility rate and the percentage of pregnant females.

On the other hand, it was found that, paternal exposure to (0.1 mg/kg body weight) of folic acid did not affect the fertility rate where all the females in the breeding group were recorded pregnant when compared with the control.

3.2.4- In vivo effect of 5-azacytidine and folic acid treatments on the mice females' pregnancy –out come:

An investigation was carried out to study the effects of pregnant mice females exposure to either (8 mg/kg body weight) of 5-azacytidine or (0.1 mg/kg body weight) of folic acid on the embryo development and the pregnancy –out come.

Table (3.10) shows the in vivo effects of 5-azacytidine on the pregnancy –out come in a breeding group consisted of fifty pregnant mice females (25-control and 25-pregnant mice females treated with 5-azacytidine).

Table (3.10): *In vivo* effect of intraperitonial injection of (8mg/kg body weight) of 5-azacytidine on the pregnancy –out come of fifty pregnant mice females, administrated at the (7^{th} -10^{th}) days of pregnancy.

Examination items	Control females (n=25)	5-azacytidinetreated females (n=25)
Corpora lutea counts	175	175
No. of implantation site	170	170
No. of resorption	5	12
No. of viable fetuses	169	160
Preimplantation losses	5	*12
post implantation losses	1	*10

* **Preimplantation losses and post implantation losses increase.**

The results of this experiment had revealed that, the injection of the pregnant mice females with (8 mg/kg body weight) of 5-azacytidine at their (7^{th},8^{th}, 9^{th}, and 10^{th}) days of gestation was harmful to the embryos where an increase in the embryo lethality was recorded probably due to the increase in the pre-implantation and post-implantation losses recognized in the breeding group of the 5-azacytidine treated pregnant mice females when compared with the control breeding group.

On the other hand, the investigation of folic acid effect on the pregnancy –out come was determined through the oral administration of (0.1 mg/kg body weight)of this vitamin during the (0-8th) days of gestation which was followed by heat treatment for (8.5 minutes in a water bath at 43 ºC).

Table (3.11) shows the results of folic acid administration on the pregnant females following heat exposure within a breeding group of forty pregnant mice females.

Table (3.11): *In vivo* effects of oral administration of (0.1 mg/kg body weight) of folic acid on the pregnancy-out come of mice females following heat treatment for (8.5) minutes in a water bath at 43 ºC.

Examination items	Control group		Folic acid group	
	No heat (n=10)	Heat (n=10)	No heat (n=10)	Heat (n=10)
Corpora lutea count	72	71	70	73
No. of implantation site	68	69	67	71
No. of resorption	4	2	3	2
No. of viable fetuses	66	37	66	70
Preimplantation losses	4	2	3	2
Postimplantaion losses	2	*32	1	1

* **Postimplantaion losses increase.**

However, data of this table demonstrated that, the exposure of pregnant mice females to heat treatment at their eighth day of pregnancy probably had induced an increase in the rates of post – implantation losses where (32 embryos) were lost in the breeding group that received heat treatment only without the supplementation of folic acid. On the other hand, only one

embryo was lost in the breeding group where the pregnant mice females had received oral administrations of folic acid prior and after the heat treatment which probably indicated that the supplementation of folic acid prior heat treatment had reduced the deleterious effects of this treatment on the pregnancy-out come.

3.2.5-Teratogenic effects of 5-azacytidine treatment:

In this experiment, both parents were treated with (8mg/kg) of 5-azacytidine and mating colonies were constructed as in table (3.12):

Table (3.12): Treatment of mice males and females with (8mg/kg) 5-azacytidine and formation of mating colonies.

Mating colony No.	Animal/group	Mice male treatment	Mice female treatment
1	7	(8mg/kg)azacytidine	(0.1ml)D.W
2	7	(0.1)D.W.	(8mg/kg)azacytidine
3	7	(8mg/kg)azacytidine	(8mg/kg)azacytidine
4	7	(0.1)D.W.	(0.1)D.W.

Results of these mating colonies were shown in table (3.13):

Table (3.13): Teratogenic effects of 5-azacytidne on mice pregnancy Out-comes and new borne.

Mating colony number	Pregnancy out-come
1	Normal
2	*Malformed
3	All dead
4	Normal

However, figure (3.17) shows the result of t5he treatment of both parents with 5-azacytidine where a malformed new borne resulted with macrocephaly, cleft palate and abnormal limbs which did not survive for more than two days.

Figure (3-17): teratogenic effect of 5-azacytidie on mice

3.2.5- In vivo effects of 5-azacytidine and folic acid treatments on the chromosomes of the mouse bone marrow cells:

This experiment was carried out in order to investigate the effects of the administration of either 5-azacytidine or folic acid on the chromosomes of the somatic cells of the treated mice bone marrow. Results shown in figure (3.18) indicated that, there was a significant increase ($p < 0.05$) in the mitogenic activity of the chromosomes of the treated mice bone marrow somatic cells when compared with the mitogenic activity of the control group.

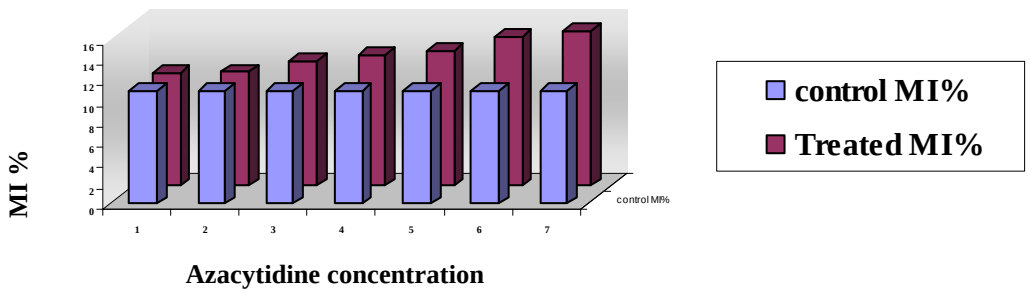


Figure (3.18): *In vivo* effect of intraperitonial administration of (8 mg/kg body weight) of 5-azacytidine on the mitotic activity (MI %) of the chromosomes of the treated mice bone marrow somatic cells *(Mean ± SD)*.

On the other hand, results shown in figure (3.19), which were obtained following the oral administration of (0.1 mg/kg body weight) of folic acid revealed a non-significant increase($p > 0.05$) in the mitotic activity of the chromosomes of the bone marrow somatic cells. In addition, no morphological abnormalities were detected in the examined chromosomal preparations taken from the treated animals with folic acid.

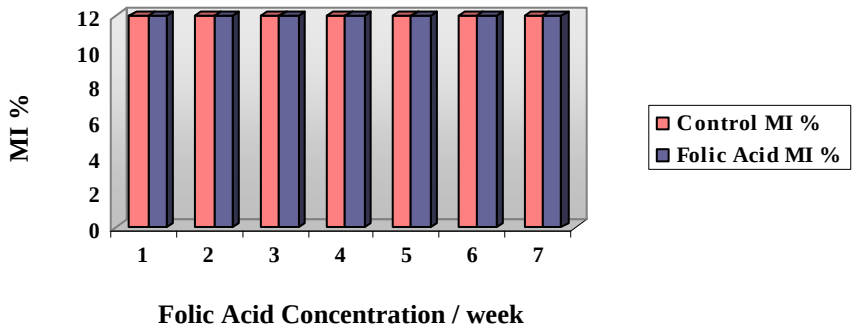


Figure (3.19): *In vivo* effect of (0.1 mg/kg body weight) of folic acid oral administration on the mitotic activity (MI %) of the chromosomes of the treated mice bone marrow somatic cell.

3.2.6-In vivo effects of 5-azacytidine and folic acid treatments on the genomic DNA methylation pattern and the cytoplasmic protein content of the mouse liver:

3.2.6.1- The effect on the genomic DNAmethylation pattern of the liver cells in vivo :

A-DNA isolation from mouse liver:

Genomic DNA was extracted and purified from mouse liver cells according to the procedure described by Sambrook *et al.*, (1989). Suitable quantities of approximately (120-150 mg/ml) of DNA were isolated from about (1 g) of liver tissue. DNA concentrations and purity measurements were estimated by measuring the absorbance of the DNA from each liver sample at (269-280 nm) wave lengths. Results were shown in table (3.14).

Table (3.14): Spectrophotometer measurements of DNA concentrations and purity of four liver samples where (sample 1=control, sample 2=5-azacytidine, sample 3=folic acid) treated mice livers.

Samples	A1 (260 nm)	A2 (280 nm)	Purity A1/A2	DNA conc. mg/ml	Protein conc. mg/ml
Sample 1	0.865	0.483	1.79	37.037	94.527
Sample 2	0.675	0.412	1.637	27.597	128.85
Sample 3	0.86	0.48	1.78	36.83	93.998

It could be noticed from this table that, the ratio between the readings at 260nm and 280nm, (OD260/OD280) which provided an estimation of the purity in the liver samples had the values of (1.79) for the control sample and (1.78) for the folic acid sample respectively. Whereas, the DNA purity values of the 5-azacytidine sample was (1.64), which was lower than the values of the control and folic acid samples probably, because the DNA was heavily contaminated with (5-azacytidine) substances that might absorbed the Ultraviolet irradiation and therefore impeded accurate analysis (Sambrook *et al.,* 1989).

B - DNA digestion with (HpaII and MspI) restriction enzymes:

Equal concentrations of DNA's from the three liver samples were digested with (5 µl) of each enzymes of HpaII or MspI. The digested products were analyzed on a gel of (1.6%) of agarose concentration and the results were shown in figure (3.20).

λ	C	CH	CM	A	AH	AM	F	FH	FM

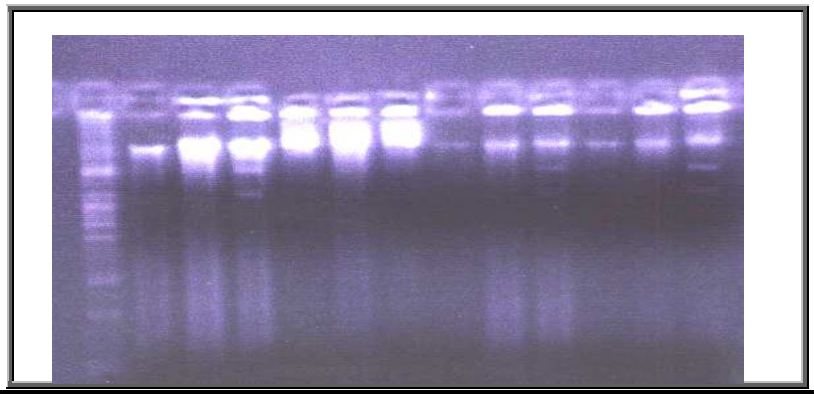


Figure (3.20): Gel pattern of mouse liver DNA (10 µg) treated with either HpaII or MspI restriction enzymes and incubated over night at 37 ºC. Where, (lane C)= un cut control DNA, (lane CH)= control DNA + HpaII (2units)), (lane CM)= control DNA +MspI(2 units)), (lane A)= un cut 5-azacytidine DNA, (lane AH)= 5-azacytidine DNA + HpaII (2 units)), (lane AM)= 5-azacytidine DNA + MspI (2units)), (lane F)= un cut folic acid DNA, (lane FH)= folic acid DNA + HpaII (2 units)), (lane FM)= folic acid DNA + MspI (2 units) with DNA marker of lambda DNA PstI digest., Mixtures were stained with ethidium bromide and photographed.

Careful examination of this figure showed the following results:

1- Lanes (C& F) showed single DNA fragments with a molecular size of (5.077 kb) where (lane C) represented the uncut DNA of the control liver sample and (lane F) represented the uncut DNA of the folic acid treated liver sample.

2- Whereas, (lane A) that represented the uncut DNA of the 5-azaytidine treated liver sample showed the distribution of two DNA fragments with the molecular sizes of (11.497 and 5.077 kb).

3- Moreover, lanes (CH & FH) showed the distribution of two DNA fragments with the molecular sizes of (11.497 and 5.077 kb), digested with (2 units) of HpaII methylation sensitive restriction enzyme.

4- While, (lane AH) showed the distribution of a fragment with a molecular size of (11.497 kb) with another smeary fragment of a probable molecular size of (5.077 kb).

5- On the other hand, lanes (CM & FM) showed the distribution of three DNA fragments with the molecular sizes of (11.497, 5.077 and 4.507 kb) produced after DNA digestion with (2 units) of the methylation non-sensitive MspI restriction enzyme.

6- While, the same distribution of DNA fragments that was recognized in (lane AH) was also detected in (lane AM).

However, table (3.15) shows the results of digestion with HpaII and MspI restriction enzymes and the size distribution of the DNA fragments more clearly as follows:

Table (3.15): The size distribution of DNA fragments digested with HpaII and MspI for DNA samples isolated from livers treated with (5-azacytidine A and folic acid F) compared with the DNA of the control (C), (+)= presence of the fragment, (-)= absence of the fragment

Marker Lambda Pst I "bp"	Control lanes			5-azacytidine lanes			Folic acid lanes		
	C	CH	CM	*A	AH	*AM	F	FH	FM
11.497	-	+	+	+	+	+	-	+	+
5.077	+	+	+	+	+	+	+	+	+
4.749	-	-	+	-	-	-	-	-	+
4.507	-	-	+	-	-	-	-	-	+

*Differences in the restriction enzyme digestion pattern.

C-Randomly amplified polymorphic DNA-polymerase chain reaction analysis"RAPD-PCR":-

In this study, six random primers of decamer oligonucleotides were screened for RAPD-PCR analysis of the predigested DNA's with HpaII and MspI restriction enzymes. Table (3.16) shows the sequence of the primers used.

Table (3.16): Sequence and GC –ratio for a number of decamer primers used in RAPD-PCR analysis

GC ratio	Sequence	Primers
6:10	5-TGCCGACACTG-3	OPA-02
6:10	5-TCGGCGATAG-3	OPA-12
7:10	5-CAGCACCCAC-3	OPA-13
6:10	5-CCTTGACGCA-3	OPB-12
7:10	5-ACCCGGCTCAC-3	OPD-20
6:10	5-AGATGCAGCC-3	OPE-7

However, results obtained from the RAPD-PCR analysis using these primers were classified into two categories:

In the first category, no amplified products were detected, inspite of repeating the number of experiments using different primers (OPA-02, APA-12, OPA-13, and OPB-12 & OPD-20), the same results were obtained.

On the other hand, the second category of RAPD – PCR amplification products were obtained using the primer (OPE-7), where a reasonable degree of DNA polymorphism was detected among the four liver DNA samples as it was manifested by figure (3.21).

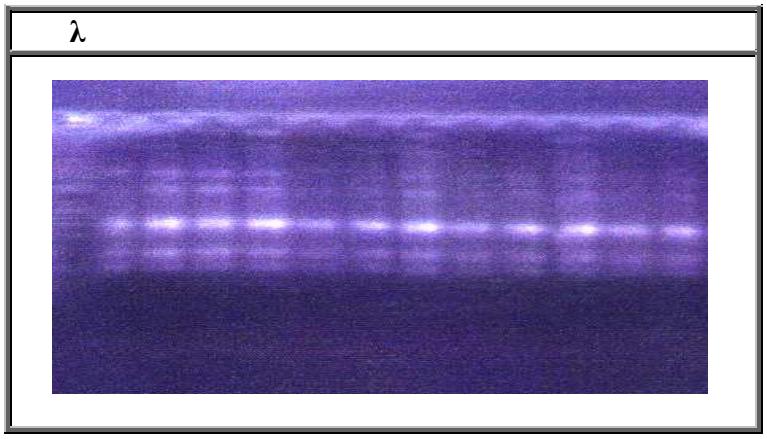


Figure (3.21): Amplification products of RAPD – PCR using primer OPE-7 of DNA samples digested with HpaII and MspI restriction enzymes.Where, (lane C)= un cut control DNA, (lane CH)= control DNA + HpaII (2units)), (lane CM)= control DNA + MspI (2 units)), (lane A)= un cut 5-azacytidine DNA, (lane AH)= 5-azacytidine DNA + HpaII(2 units)), (lane AM)= 5-azacytidine DNA + MspI (2units)), (lane F)= un cut folic acid DNA, (lane FH)= folic acid DNA + HpaII (2 units)), (lane FM)= folic acid DNA + MspI (2 units) with DNA marker of lambda DNA PstI digest. Mixtures were stained with ethidium bromide and photographed.

Moreover, the results detected in this figure showed three forms of banding pattern analyzed according to Williams *et al.*, *(*1990) as follows:

1. The presence or absence of DNA bands.
2. Difference in the molecular size of the bands.
3. Difference in the intensity of the amplified bands.

Table (3.17) describes the results more clearly:

Table (3.17): The size distribution of DNA fragments pre-digested with HpaII and MspI restriction enzymes and amplified with RAPD –PCR using the primer OPE-7

Marker Lambda PstI "Kb"	Control lanes			Azacytidine lanes			Folic acid lanes		
	C	CH	CM	A*	AH*	AM*	F	FH	FM
11.497	-	-	-	-	-	-	-	-	-
5.077	+	-	-	-	+	+	+	-	-
4.749	+	-	-	+	+	+	+	-	-
4.507	+	-	+	+	+	+	+	-	+
2.838	+	+	+	+	+	+	+	+	+
2.560	+	+	+	+	+	+	+	+	+
2.459	+	+	+	+	+	+	+	+	+
2.443	-	-	-	+	+	-	-	-	-

*Polymorphism.

3.2.6-Effects of the treatments on mouse liver cytoplasmic protein contents:

The comparison between the cytoplasmic protein contents of the liver samples treated with (5-azacytidi, methotrexate and folic acid) was allowed using (SDS-PAGE) shown in figure (3.22).

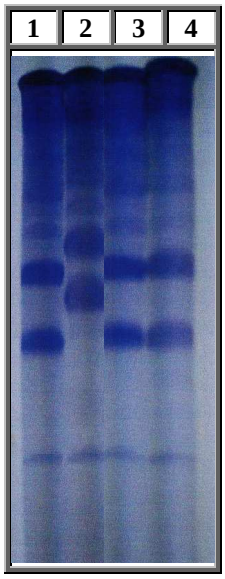


Figure (3.22): SDS-PAGE of mice liver cytoplasmic protein samples where, (lane 1) = control, (lane 2)= 5-azacytidine treated liver sample, (lane 3)= folic acid treated liver sample treated liver sample.

Results of this figure suggested that, products of (lane 1) electrophoresis had represented the normal phenotypic composition of the mouse liver cytoplasmic proteins where this lane represented the untreated liver sample.

While, electrophoresis products of (lane 2) showed a quantitative increase in a set of two middle bands with a slight variation in the migration rate or (Rm-value) of other bands.

A remarkable similarity was detected in the banding pattern of the cytoplasmic proteins of (lane 3) suggesting that, the gene expression of the liver DNA was not probably affected by these treatments. Therefore, the same phenotypic composition of the cytoplasmic proteins were found in the liver samples treated with folic acid.

However, table(3.18) was suggested according to Comings and Tack(1972)in which it was shown the difference in the migration rates or (Rm-values) of the cytoplasmic proteins detected by the sodium dodecylsulphate polyacrylamidede gel technique.

Table (3.18): The migration rates of the cytoplasmic proteins of mice liver samples detected by SDS-PAGE, where (lane 1)= control, (lane 2)= 5-azacytidine, (lane 3)= folic acid cytoplasmic proteins.

Band no.	Lane1	Lane 2	Lane3
1	4	4	4
2	8	*10+ 12	8
3	16	16	16
4	24	28	24
5	32	32	32
6	42	38	42
7	56	56	56
8	70	70	70

***Additional two middle bands.**

Summary

***In vitro* and *in vivo* effects of 5-azacytidine on some developmental aspects in mice.**

Table (3.19): In vitro effects of 5-azacytidine on somatic mouse cells.

Treated cell cultures assays	Results
Fibroblast cell-short term viability	Cytotoxic
Fibroblast cell-long term survival	Decrease plating effeciency
Fibroblast cell –differentiation state	Affect cellular phenotype
Plasmacytoma –short term viability	Cytotoxic
Plasmacytoma long term survival	Decrease plating effeciency
Plasmacytoma differentiation state	Induce reverse mutations and show hermetic effect on exposed cells.

Table (3.20): In vivo effects of (8mg/kg) of 5-azacytidine in mice.

In vivo system	Results
#Male	**Affected by the treatment**
Body weight	Significantly reduced
Testes histology	Histological abnormalities
Sperms	Functional &morphological abnormalities
Fertility rate	Reduced fertility rates
Chromosomes	Increased mitotic activity
#Females	**Affected by the treatment**
Body weight	Significantly reduced
Pregnancy out-come	Reduced &embryonic abnormalities
Teratogenic effects	Malformed new borne
#Liver cells	**Affected by the treatment**
DNA analysis	Hypomethylating &genotoxic effects
protein analysis	Drifted polypeptide chain migration rates

Chapter Two

Materials & Methods

2.1- Materials:

2.1.1- Equipments: The following equipments were used throughout the work:-

Equipment	Company	Country
Autoclave	Tommy	Japan
Balance (Analytical)	Mettler	Switzerland
Bench – centrifuge	M / W	Germany
Cooled incubator	Sanyo	Japan
Distillator	Gallenkamp	England
ELISA reader	Organon	Austria
Gel documentation unit	Consort	Belgium
Gel electrophoresis (horizontal) tank	Flowgen	England
Inverted microscope	Microstar	U.S.A.
Compound Light microscope	Olympus	Japan

Equipment	Company	Country
Compound Light Microscope with Camera	Olympus	Japan
Laminar flow hood	Nap Flow	France
`Microfuge	Eppendorf	Germany
Microtome	Leica microsystem	Germany
Magnetic stirrer Hot plate	Ceramagemidi IKA	U.S.A.
Millipore filter unit	Bed ford mass	U.S.A.
cycle personal (PCR unit)	Eppendorf	Germany
Oven	Gallen kamp	England
Polyacrylamide gel Polymerization pack	ISCO	U.S.A.
Polyacrylamide Vertical electrophoresis chamber	ISCO	U.S.A.
Power supply	Techware 257/2	Germany
Refrigerator	NRC	Jordan
Refrigerated centrifuge	Harier 18/80	England
Sterio microscope	Will DM3	Switzerland
Sensitive balance	A&D Ltd.	Japan
Spectrophotometer	Schimadzo	Japan
U.V. transilluminator	UVITEC	England
Water bath	Memmert	Germany

2.1.2- Chemicals:

The following chemicals were used during the work:-

2.1.2.1- BDH - England:

Ammonium persulfate, Crystal violet, Disodium phosphate, Ethanol, Glacial acetic acid, Glycerol, Glycine, Neutral red, Sulphoric acid, Sucrose, Sodium azide, Sodium dodecyl sulfate (SDS), Sulphossalicylic acid, Trichloric acid, Triethanol amine, Trisbase, Tris-HCl.

2.1.2.2- Fluka - Switzerland:

Acetic acid, Boric acid, Chloroform, Calcium chloride, Dithiotheritol (DTT), Hydrochloric acid, Methylene blue, Magnesium chloride, Mercury oxide, Potassium chloride, Potassium hydroxide, Potassium dihydrogen phosphate .

2.1.2.3- LKB - Sweeden:

Acylamide, N, N –methylenbisacrylamide.

2.1.2.4- Operon – U.S.A:

Primer OPA – 13, Primer OPA – 12, Primer OPB – 12, Primer OPD – 20, Primer OPE – 7.

2.1.2.5- Promega – U.S.A:

Taq DNA polymerase, Polymerase chain reaction buffer.

2.1.2.6- Samara drug industry(SDI) – Iraq:

Amphotrecin, Benzyl – penicillin, Streptomycin.

2.1.2.7- Sigma – U.S.A:

5- azacytidine, Agarose (type 1-B Low), Bromophenol blue – xylene cyanole dye, Colcemide, Bromocresol purple, Coomasie brilliant blue R – 250, Deoxynucleotide phosphate mixture, Disodium – ethylendiamine tetra acetic acid, Ethylene diamine bromide, Eagle's medium powder, Fetal calf serum, Folic acid, Formalin, Giemsa stain powder, Glucose, Glycerin, HEPES, Hypoxanthine aminopterine thymidine (HAT) medium powder, Hemo-dye powder, Methanol, Medium 199 powder; N,N,N,N–

2.1.5.5.1- Giemsa stain solution:

This stain was prepared according to the procedure mentioned by (AL-Attar *et al.*, 1982). Giemsa stock solution was prepared by dissolving (1g) of Giemsa powder in (3ml) of glycerin. The mixture was placed in water bath at (60 ºC) for 2 hours with continouse shaking. Then, (66 ml) of absolute ethanol was added to the mixture and the stock was stored in the dark bottle at room temperature.

For slide staining, Giemsa stock solution was diluted as follows:

About (1 ml) of Giemsa stock solution was mixed with (1.25 ml) of absolute methanol, (0.11g) of sodium bicarbonate and (40 ml) of distilled water. Then, the mixture was filtered through Watman no.1 filter paper and used immediately in staining.

2.1.5.5.2- Neutral red dye solution (0.4%):

This dye solution was prepared according to (Freshny, 1994) by dissolving (0.01 mg) of neutral red powder in (100 ml) of distilled water. Then, this dye was either used directly in the short-term viability assays or stored at room temperature.

2. 1. 6- Solutions used in the animal experiments of the study:

2.1.6.1- The solution of 5 - azacytidine (8mg / kg mouse body weight):

This solution was prepared according to (Constaniniedes *et al.*,1978)by dissolving (0.2mg) of 5-azacytidine powder in (0.1ml) PBS, sterilized by filtrations and used immediately for intraperitonially injection of the experimental animals.

2.1.6.2- The solution of folic acid (0.5 mg/kg mouse body weight):

This solution was prepared according to (Hernandez-Diaz *et al.*,2000) by dissolving (0.125mg) of folic acid in (0.1ml) of distilled water, sterilized by filtration and used immediately for the treatment of the experimental animals.

2.1.6.4- Cytogenetic assay solution:

2.1.6.4.1- Colcemid solution (0.6%):

This solution was prepared according to (Freshney, 1994) by dissolving (0.6g) of colcemid in (100 ml) of HBSS stirred to dissolve and sterilized by filtration, dispensed into aliquots of (20 ml) and were stored at (4˚C).

2.1.6.4.2- Phytoheamagglutinine (PHA) solution:

The PHA solution was prepared according to (Freshney, 1994) by dissolving (500 mg) of PHA powder in (100ml) HBSS, sterilized by filtration and dispensed into aliquots of (20 ml) and were stored at (4˚C).

2.1.6.4.3- Hypotonic solution (0.075M):

This solution was prepared according to (AL-Attar *et al*.,1982) by dissolving:

KCL	1.1175 g
D.W	200 ml

The stock solution was stored at (4 ºC).

2.1.6.4.4- Fixative solution:

This solution was Freshly prepared according to (AL-Attar *et al*., 1982) and was made as a mixture of the following:-

Methanol	3 parts
Glacial acetic acid	1 part

The pH of (Tris- base and EDTA) was adjusted to (8.0) then mixed with the other compounds.

2.1.7.2- Protinase K stock solution:

This stock solution was prepared by dissolving (20 mg) of protinase K powder in (1 ml) of distilled water and stored at (-20 ºC).

2.1.7.3- Phenol: chloroform solution:

A mixture consisted of equal parts of phenol and chloroform (1:1) was prepared by mixing (250 ml) of phenol with (250ml) of chloroform and the mixture was stored at (4 ºC) in a dark bottle and used in a month.

2.1.7.4- TE buffer (pH8.0):

Prepared by dissolving about (0.39g) of Tris-base and (0.73g) of EDTA in (250ml) of distilled water stored at (4 ºC).

2.1.8- Restriction Endonuleases –DNA digestion mixture:

According to (Singer-Sarm *et al.,* 1989) the following materials were mixed as follows:-

Reaction mixture components	Concentration / reaction	Volume / reaction
HpaII enzyme	1U / 1µg DNA	1.2 µl
MspI enzyme	1U /1µg DNA	1.2 µl
DNA Samples	10 µg	5.5 µl
Restriction buffer	5%	4 µl

2.1.9- Buffer and stain used in agarose gel electrophoresis:

The following solutions were prepared according to the method mentioned by (Sambrook *et al.,* 1989) as follows:

This mixture was stirred on a magnetic stirrer for several hours to dissolve the dye, and then dye was placed in a container warped with aluminum foil and stored at room temperature.

2.1.10- RAPD – PCR amplification reaction components:

2.1.10.1- Reaction master mix:

The following components were mixed together in a (50 µl) eppendorf tube according to (Sambrook, *et al.,* 1989).

Reaction component (Master mix)	Concentration in the reaction	Volume in reaction
PCR-buffer pH (7.2)	10 X	2.5 µl
dNTPs	10 µl	0.5 µl
Taq DNA polymerase	2U	1.5 µl
Primer	10 p mol	1 µl
DNA sample	25 µg	(dependent on DNA concentration and sample purity)

2.1.10.2- Primers used in PCR reactions:

The primers used in the RAPD – PCR amplification reaction had the following sequences according to Operon company:-

Primers used	DNA sequences
OPA-02	5-TGCCGAGCTG-3
OPA-12	5-TCGGCGATAG-3
OPA-13	5-CAGCACCCAC-3
OPB-12	5-CCTTGACGCA-3
OPD-20	5-ACCCGGTCAG-3
OPE-7	5-AGATGCAGCC-3

2.1.10.3- Agarose gel (1.2%):

This gel was prepared for the analysis of PCR- amplification products by dissolving (0.48g) of agarose powder in (40 ml) of TBE buffer. The slurry

2.1.11.10- Sample buffer (A):

This buffer was prepared according to (Laemmli, 1970) by dissolving the following: -

Sucrose	8 g
Potassium chloride	0.22 g
Magnesium chloride	0.2 g
Dithiotheritol (DTT)	0.015 g
D.W.	100 ml

2.1.11.11- Sample buffer (B):

According to (Janson and Ryden, 1998), this buffer was prepared by mixing the following:

SDS	2 g
DTT	7.5 g
D.W.	100 ml

2.2- Methods:

2.2.1- Sterilization methods:

Sterilization methods were carried out according to (Levinson, 2004) as follows:

2.2.1.1- Dry heat(Oven sterilization):

A laboratory oven was used for the sterilization of glass wares and dissection sets at (200 ºC) for 2 hours.

2.2.1.2 – Steam sterilization(Autoclave):

Buffers and certain solutions were sterilized using the pressure vessel (autoclave) at (121 ºC) and (15 pounds / in^2) for 15 minutes.

2.2.1.3- Filter sterilization:

Millipore filter unit with (0.45 µm) filter paper was used for the sterilization of media, enzymes and some agent like (5-azacytidine, methotrexate and folic acid).

2.2.2- Management of laboratory animals:

Healthy, adult mice animals of ***Swiss albino*** strain were obtained from the animal house of Research center for Biotechnology of Al-Nahrain University. Five hundreds of mice animals were used in this study (300 females and 200 males), ranging in age between (8-12) weeks old and their weight were about (23- 25g).These animals were kept in an air condition room at a temperature of (22-24 ºC),with about (12 -14) hours of day light exposure. Animals were housed in cages measuring (29 * 15 * 12 cm) and each seven animals were kept in one cage contained wooden shave. Water and Feed composed of (wheete, barely mixed with 250mg of milk powder) were freely excess able and animals were kept for at least two weeks for adaptation.Animal cages were cleaned and sterilized with 70% ethanol once a week regularly according to the procedure mentioned by (Small, 1983).

2.2.3- Vaginal smears:

Estrous cycle was diagnosed by repeated vaginal smears according to the procedure of (Hafez, 1987) as follows:

i. One drop of sterile normal saline (8.5%) was placed by loop in the vagina, aspirated several times then, the mixture was transferred to clean slide.

ii. Few drops of (0.05%) of methylene blue dye prepared by dissolving (0.05g) of methylene blue in (100 ml)distilled water was placed over the slide dried and washed with tap water then, slides were examined under light microscope (40 x).

iii. Smears were performed daily between (9 A.M.) to (12 o’ clock) at noon for at least two cycles.

ii. Cells were seeded in (10^4 cell/ml) in tissue culture flasks containing (5 ml) Eagle's medium.

iii. Then, 5- azacytidine was added in concentrations of (1, 3 and 5 mM) and flasks were incubated at 37˚C for 24 hours.

iv. Cells were washed after the Eagles medium was added and (2 ml) of fresh Eagle's medium was added and flasks were re- incubated at 37˚C for (14 days) with medium changed twice a week

v. Flasks were stained with Giemsa and examined for any morphological changes.

2.2.7.3- Production of HAT – resistant plasmacytomas sub - clones:

The following procedure was described by (Mohandas *et al.,* 1981) as follows:

i. Plasmacytoma cells were mechanically harvested, centrifuged and resuspended in (5 ml) of HAT - medium at a cell density of (10^3 cell /ml) incubated for three days at 37˚C and 5% CO_2.

ii. Another cell suspension was prepared in a cell density of (10^3 cell/ml) and cells were suspended in (5 ml) of RPMI 1640 medium and flasks were incubated at 36.5 ˚C for 24 hours.

iii. The agent 5- azacytidine was added to the culture in concentration of (0, 1, 3, 7, 9 μM) and cultures were incubated at 37˚C for (24 hours).

iv. Cells were washed with sterile PBS and (5 ml) of RPMI 1640 medium was added and flasks were re-incubated at 37˚C for three days.

v. Then, medium was changed to (5 ml) of HAT- medium and cells were further incubated for 2 weeks at37˚C with medium change twice a week.

vi. After 2 weeks of incubation, flasks were washed with PBS, fixed with methanol, stained with Giemsa stain and photographed.

2.2.8- In vivo treatments of the white mice:

2.2.8.1- Treatment of white mice with 5- azacytidine:

i. Forty adult males weighed (24-25g) and females weighed (22-23g) of ***Swiss albino*** strain were selected, ranged in age between (8-12) weeks old.

ii. Animals received intraperitonial injections of (0.1 ml) of, PBS (n= 10 mice / group) or of (8, 10 mg/kg) of 5-azacytidine (n = 7 mice /group), three times a week for 6 weeks according to (Poirier, *et al.*, 2003).

iii. The LD_{50} of 5- azacytidine was defined as the dose that caused the death of 50% of the animal in the assigned treatment group according to Livenson (2004).

2.2.8.2- Treatment of white mice with folic acid:

i. Forty adult males weighed (24-25g) and females weighed (22-23g) of ***Swiss albino*** strain were selected, ranged in age between (8-12) weeks old.

ii. Animals were administrated orally with (0.2 ml) of, distilled water (n=10 mice /group) or of (0.1, 0.5 mg /kg) of folic acid (n=7 mice /group), three times a week for 6 weeks according to (Poirier, *et al.*, 2003).

iii. The LD_{50} of folic acid was defined as the dose that caused the death of 50% of the animals in the assigned treatment group according to Livenson (2004).

2.2.9- Studying the effects of the in vivo treatments on the white mice:

2.2.9.1- In vivo effect on the white mice body weight:

The following procedure was mentioned by (Jackson-Grusby and Jaenisch, 1996).

i. About (7) animals of both sexes were randomly selected for the investigation of the treatment effect on the mouse body weight.
ii. Initial body weight of each animal was recorded at the day zero of the treatment.
iii. Body weights were recorded at the end of each treatment week by weighing each animal separately on a balance.
iv. Initial and final body weights were compared together with the control groups and their significant differences were determined.

2.2.9.2- In vivo effect on male's reproductive organs:

This experiment was mentioned by (Doersken *et. al* ., 2002) and about (10 male) animals were randomly selected from each group and the procedure was as follows:

2.2.9.2.1- Disection of testes:

i. Five male animals were randomly selected from each treatment group and were sacrificed by cervical dislocation and their venteral surface was swabbed with 70% ethanol.

xii. Analysis of abnormal somniferous tubule cross sections was performed using a serious of photographs of testicular cross-sections from each animal.

xiii. The numbers of morphologically normal and abnormal tubule cross- sections were counted and expressed as percentage.

2.2.9.2.3- Sperm collections:

The following procedure was mentioned by (Hinting, 1989) as follows:-

i. Five male animals from each treatment group were placed with untreated females one day before killing.

ii. Males were killed in the next days by cervical dislocations, then the ventral skin was swapped liberally with 70 % ethanol then the skin was torn at the media line and the skin was grasped on both tears sides in an opposite directions exposing the male reproductive organs to the out side.

iii. About 2-3 cm length of the epididymus was cut and transferred to Petri dishes contain 3 ml of PBS.

iv. Sperms were collected by pushing 5 ml of RPMI medium through one end of the epididymis with a sterile syringe and collecting the sperms in a petridishes.

2.2.9.2.4- Microscopic examination of the sperms:

2.2.9.2.4.1- Sperm motility:

The following procedure was mentioned by (Mortimer *et. al*, 1988) as follows: -

i. A drop of 50 µl of sperm suspension was immediately placed over a warm slide after sperm collections.

ii. The slides were quickly examined at (40 x) objective of a light microscope and five random filed of the slides were scored for the examinations.

iii. The percentage of the abnormal sperms was assessed according to the following table:-

Sperm motility percentage	Description
0 %	Non motile
(0-20)%	Some sperms are motile
(20-45)%	High number of non motile sperms
(45-55)%	Half are motile
(55-85)%	High number of motile sperms
(85-100)%	Most sperms are motile

2.2.9.2.4.2- Sperm concentration:

Sperm concentrations were calculated according to the procedure mentioned by (Hafez, 1987):

a. A drop of (50 µl) of sperm suspension was placed over a warm slide and covered with a cover slip.
b. The concentration of sperms was calculated by examining five randomly selected fields under (40x) objective of a light microscope.
c. The calculated number was multiplied by a factor of one million to obtain the (sperm concentration/µl).

2.2.9.2.4.3- Sperm viability:

The following procedure was described by (Hafez, 1987) as follows:

a. A drop of (50 µl) of sperm suspension was placed over a slide.
b. Another drop of (50 µl)of eosin – stain was mixed with the sperm suspension and examined under the (100x) objective of a light microscope, and 200 sperm were counted to calculate the percentage of dead sperm according to the following equation:

$$\text{Percentage of dead sperm} = \frac{\text{No. of dead sperms}}{\text{Total No. of sperms}} * 100$$

2.2.9.4.2- Treatment of pregnant females with (0.1mg/kg) of folic acid:

This procedure was carried out according to (Shin and Shiota, 1999) as follows:-

a. Ten females of eight weeks old and of (22-23g), were mated over night with active male and were examined in the next morning for vaginal plugs.
b. Pregnant females were divided into two groups: the first group was orally fed with folic acid (0.1mg /kg) and the treatment was started from the first day of pregnancy and lasted for (10days) of pregnancy.
c. The control group received (1ml) of distilled water only.
d. Heat treatment was induced at the (8th day) of pregnancy by placing the pregnant females in a water bath at 43ºC for (8.5 minutes).
e. Half the females from each group received heat treatment while the other half did not receive any heat treatment.
f. Treated animals were sacrificed at their (20^{th} day) of pregnancy for the same counts mentioned in (2.2.9.4.1-iv).

2.2.10- Cytogenetic analysis of bone marrow somatic cells:

The following procedure was carried out according to (Allen *et al.*, 1977) as follows:

2.2.10.1- Preparation of the chromosomes:

i. Males were injected with (0.25 ml) of colchicine intraperitonially two hours before sacrificing.
ii. Males were sacrificed by cervical dislocation on their ventral side on an anatomy stage.
iii. Both the abdominal side and the thigh region of the animal were swabbed with 70% ethanol.

iv. Then, the thigh bone was taken and cleaned from other tissues and muscles, then gapped from the middle with a sterile forceps.

v. The bone was held in a vertical position and the bone marrow was collected by injecting the bone with (5ml) of PBS using a sterile syringe.

vi. A hypotonic solution was added in (5 ml) and cells were incubated at (37 ºC) for 30 minutes.

vii. Cells were centrifuged and a fixative was added and tubes were kept at (4 ºC) for 30 minutes.

viii. Tubes were centrifuged at (1000 rpm) for 10 minutes and cells were resuspended in (2 ml) of a fixative.

ix. Few drops were dropped vertically on a cold, clean slide from a height of three feet at a rate of (4-5) drops; slides were left to dry, then stained with Giemsa.

x. Slides were examined under the (100 x) objective of a light microscope and three slides were made for each animal.

2.2.10.2- Mitotic index:

The slides were examined under the (40 x) objective and about (1000 cells) of divided and non - divided were counted, the mitotic index was calculated according to the following equation:

$$\text{MI \%} = \frac{\text{N0. of divided cells}}{1000} * 100$$

2.2.11- Isolation of DNA from mouse liver samples:

The following procedure was carried out according to (Sambrook *et al.*, 1989) with some modifications designated by * as follows:

i. *A weight of (1g) of mouse liver was finely chopped with sterile forceps and washed several times with PBS.
ii. *Then, chopped, washed liver tissues were transferred into eppendorf tubes and were centrifuged at 5000 rpm for 10 minutes at room temperature.
iii. *To the precipitate, 10 volumes of extraction buffer were added and shake gently to submerge the materials.
iv. The mixture was then transfered to a (50 ml) centrifugation tube and was incubated for (1 hour) at 37 ºC in a water bath.
v. Protinase k was added in a final concentration of (100 mg/ml) and the enzyme was gently mixed.
vi. The suspension was placed in water bath at 37 ºC and was incubated for over night.
vii. An equal volume of phenol / chloroform was added and gently mixed for (10 minutes).
viii. Centrifugation was followed at 5000 rpm for 15 minutes at room temperature.
ix. The viscous aqueous phase was transferred with a micropipette to an eppendorf with a micropipette to an eppendorf tube.
x. Ethanol was added in (2 volumes) and the mixture was thoroughly mixed and left in freezer over night.
xi. The DNA was collected by centrifugation at (5000 rpm) for 5minutes at room temperature.
xii. The DNA was dissolved with TE.
xiii. The concentration of DNA was quantified through spectrophotometer

2.2.11.2- Spectrophotometric quantitation of DNA concentration:-

This method was mentioned by (Sambrook *et al.*, 1989):

a. About (5µl) of DNA dissolved in TE was withdraw and transfered into the Spectrophotometric cuveit with (495 µl) TE.
b. Readings were taken 260 and 280 nm wave length.
c. A ratio between the readings was obtained.
d. The concentration of DNA in (µg / µl) was calculated depending on the relation of :

(O.D. 260)*Dilution Factor*(50 µg / µl)= (µg / µl) of DNA.

2.2.11.3- Predigestion of DNA with restriction enzymes (HpaII and MspI):

The following procedure was mentioned by (Singer *et al.*, 1979).

i. About (10 mg) of DNA was digested with both enzymes (each enzyme alone) after the addition of the restriction buffer in a reaction volume of (50 µl).
ii. The reaction mixture was incubated overnight at 37 ºC in a water bath.
iii. Enzymes were inactivated by keeping the mixture on ice overnight.
iv. Digestion differences were analyzed on agarose gel (1.6 %).

2.2.11.4- Agarose gel electrophoresis:

The following procedures were carried out according to (Sambrook *et al.*, 1989).

2.2.11.4.1- Preparation of an agarose gel:

i. 2.2.12.6-The edges of a clean, dry glass plate was sealed with a tap and placed on a horizontal section of the bench.

2.2.12.6-Calculation of polypeptide chain migration rate(Rm-value):

The migration rate of the polypeptide chains was measured according to the equation mentioned by Comings and Tack (1972) as follows:

$$\text{Migration rate \%} = \frac{\text{Distance moved by the protein (mm)}}{\text{Distance moved by the tracking dye (mm)}} * 100$$

Or

$$Rm = \frac{\text{Distance moved by the protein (mm)}}{\text{Distance moved by the tracking dye (mm)}} * 100$$

2.2.13- Statistical analysis:

Data were examined using ANOVA where only two groups (n=7 animals/ group) were compared according to the procedure mentioned by Gantz, (1992). The level of significance was ($p<0.05$) L.S.D. for all analysis

Conclusions

1. An embryo age of (11-11.5) days old was suitable for the production of successful fibroblast cultures.
2. Treatment of the fibroblast - like AME 11D1/2 cell cultures with 5-azacytidine at their (5^{th} passage) had affected their phenotype rather than their differentiation.
3. Treatment of plasmacytoma SU99 cell cultures with 5-azacytidine had led to their reversion to the wild type phenotype and the production of what is called the (Hormetic effect).
4. The effect of 5-azacytidine on the plasmacytoma SU99 cell cultures had provided an interesting system to study the control of B-cell function and the possible development of cancer therapy.
5. Mice male exposure to 5-azacytidine was deleterious to the germ cells and their function in fertilization and embryo development.
6. Evidences obtained from *in vivo* animal studies had supported the generalization that, the deficiency of the physiological methyl group due to the inhibition of DNA methylation by 5-azacytidine or low levels of folic acid metabolism could cause patterns of pathological alterations probably due to their effects on the genomic DNA methylation and gene expression.

7. DNA methylation plays important roles in normal germ cell development and the establishment of allele-specific imprints in tissue differentiation making it as an attractive avenue for the development of novel cancer therapy and for the determination of patient prognosis.

Many of these chemotherapeutics are compounds that induce genotoxic effects without directly damaging the DNA, such as, the cytidine analogue, (5-azacytidine) (Stopper, 1997). This cytostatic agent has been used since 1970's for the treatment of acute leukemia (IARC, 1990), and is still being used as a part of treatment regimen for a cute leukemia, breast, Lung and Kidney cancers (Goffin and Eisenhauer, 2002). However, tissue culture studies had owed much to the evidences of the adverse effects of 5-azacytidine on various developmental aspects, since cell culture models provided a well defined, relatively simple and easily manipulated experimental systems to investigate the ability of extrinsic effects to modulate DNA stability and gene expression (Hartwell,2000 and Duthie, *et al.*,2002).

The pyrimidine analogue (5-azacytidine) has been shown to have a striking range of biological effects (Holliday, 1986). It induces morphological transformation of (3T3) and (3H/10T1/2CL8) mouse cell lines to myocytes, adipocytes and chondrocytes (Jones *et al.,* 1983).It reactivates silent genes on the inactive X-chromosome (Mohandas *et al.,* 1987), and it induces the reversion at a very high frequency of a series of enzyme-deficient mammalian cell lines to the wild-type phenotype (Taylor and Jones, 1979).

However, these examples showed that, these cell cultures were not behaving as a consequence of their quite artificial environment, but their phenotype was affected by gene reactivation which was associated with a loss of DNA methylation (Holliday, 1986), which was the result of the inhibitory effect of 5-azacytidine on the DNA methyltransferase (Jones and Taylor, 1981).where it was incorporated into the DNA as a cytidine analogue, and through its subsequent chemical interaction with the DNA methyltransferases, it prevented the normal methylation of the DNA (Jones, 1986 ; Chrisman *et al* ., 1985).

On the other hand, 5-azacytidine was an excellent DNA hypomethylating agent in vivo where it has exhibited tumorigenic and teratogenic activities in several animal studies (Poirier *et al*., 2003; Meriow *et al.,* 2000). Other

studies indicated that, chronic treatment of male rate with 5-azacytidine resulted in the demethylation of sperm DNA and suggests that both spermatocytes and spermatogonia were affected by the drug exposure (Doerksen and Trasler, 1996; Doerksen *et al.*, 2000). It was found that altering DNA methylation levels during gametogenesis by 5-azacytidine was deleterious to germ cells and their function in fertilization and embryo development, since, DNA methylation plays on important role in normal germ cell development and the establishment of allele-specific imprints in the germ line (Juttermann and Jaenisch,1994; Gabbara and Bhajwat,1995).

Moreover, methylation probably causes the inactivation of numerous genes that are important in the development of most or all tumor types thus, inhibition of DNA methylation and consequent reactivation of these genes is an attractive avenue for the development of novel therapeutics (Goffin and Eisenhauer, 2002). In addition, DNA methylation shows great promise as a marker for the early detection of cancer, and DNA methylation patterns might be of use in determining patient prognosis (Strathdee and Brown, 2002).

Therefore, on the strength of these data, this study was conducted in order to:-

1- Investigate the biological development of somatic mouse cells cultured in different media.
2- Investigate the *in vitro* effects of 5-azacytidine on the biological development and the differentiation state of the cultured cells.
3- Investigate the *in vivo* effects of 5-azacytidine and folic acid on various developmental aspects in mice including :(body weight, male reproductive system, fertility rate, pregnancy out come and bone marrow chromosomes).
4- Investigate the *in vivo* effects of 5-azacytidine and folic acid on the genomic DNA methylation pattern and liver cytoplasmic proteins.

List of contents

Recommendations

1. Other experiments are required to investigate the effect of reversion of the plasmacytoma SU99 cells to the wild type phenotype caused by 5-azacytidine using methylation specific PCR - technique with specific primers ,and checking their capability of tumor induction.

2. More histological studies are required to investigate the effects of 5-azacytidine on other mice organs and tissues.

3. Further epigenetic studies are required in order to highlight the effects of other DNA methylation inhibitors on distributing the genomic integrity and altering the DNA methylation machinery.

4. For embryonic development and 5-azacytidine effects, an experiment should be planned to follow the development of the embryos daily and focusing on partial organs.

5. Running the effect of 5-azacytidine on tumor cells as well as normal cells and following gene expression and characterization of morphology transformation.

Summary

A total of (500) mice of *Swiss albino* strain, ranged in age between (8-12) weeks old and weighed about (23-25) grams were used in this study which was divided into four parts.

- In the first part, the determination of the experimental mice females' estrous cycle was included for the establishment of mating colonies and the production of embryos used in the initiation of the fibroblast cell cultures.

Embryos at different ages of (9, 11 and 13) days old were used, and the results of the morphological examinations showed that, an embryo age of (11-11.5) days old was considered to be suitable for the production of successful fibroblast cell cultures which were designated as (the fibroblast – like AME 11D1/2 cell cultures).

- The second part of this investigation dealt with studying the biological performance of three different media including: (RPMI 1640 medium, Eagle's minimal essential medium and medium 199) on supporting the growth of (the fibroblast – like and the malignant plasmacytoma SU99 cell cultures). Results of seeding efficiency values for the two types of cells grown in the three different media had shown that, the RPMI 1640 medium was the most suitable culture medium under the experimental conditions of this study in supporting the maximum growth of the examined cell cultures.

- The *in vitro* effect of 5-azacytidine on the growth and development of the fibroblast – like and the plasmacytoma SU99 cell cultures was investigated in the third part of this study.

A cytotoxic effect on the growth of the two examined cell cultures was detected, expressed by increasing the values of the optical density measurements in the short-term viability assay and by decreasing the values of the plating efficiency of the growing cells in the long-term survival assay where the fibroblast – like cell

cultures appeared to be more sensitive to the cytotoxic effect exerted by the 5-azacytidine than the plasmacytoma SU99 cell cultures.

In addition, the phenotype of the treated cells was also affected by the 5-azacytidine treatment, where the fibroblast – like cells appeared single with different sizes other than uniform monolayers. While, the treatment of the plasmacytoma SU99 cells with 5-azacytidine induced the reversion at a high rate of an enzyme deficient malignant subclones called the (HPRT-positive cells). These cells were able to grow at HAT- medium and their reversion rate was dependent on the concentration used of 5-azacytidine in the culture medium.

- The forth part of this study was on the *in vivo* effects of 5-azacytidine and folic acid on mice body weight, male reproductive organs, embryogenesis, chromosomes of the bone marrow and gene expression on the levels of genomic DNA and whole protein content of the liver.

Results showed that, treatment of mice with 5-azacytidine caused a highly significant decrease ($p < 0.001$) in the final recorded body weight values. While, six weeks exposure of male mice to (8 mg/Kg body weight) of 5-azacytidine resulted in severe abnormalities in the seminiferous tubule, including: degeneration of the tubule, sloughing of immature germ cells into the lumen, and giant cell formation. In addition, a decrease in the fertility rate was also detected in the treated male mice revealed by the deleterious effects on some sperm's functions, including: a decrease in the motility, concentration and viability with an increase in the percentage of morphologically abnormal sperms.

Moreover, the administration of 5-azacytidine to pregnant females caused teratogenic effects where an increase in the preimplantation losses and a decrease in the pregnancy out come were detected.

Furthermore, a significant increase ($p < 0.05$) in the mitotic index with some chromosomal aberrations were also detected in bone marrow preparations from mice treated with 5-azacytidine.

On the other hand, results obtained following treatment of mice with (0.1mg/Kg body weight) of folic acid showed a non - significant increase ($p > 0.05$) in the treated animals body weights.

Moreover, it was found that, the oral administration of folic acid had no detectable effects on the treated mice males' reproductive organs or on the bone marrow chromosomes. Whereas, results obtained following treatment of pregnant females with folic acid had suggested that, prenatal supplementation of folic acid could reduce embryo lethality induced by heat exposure and sufficient folic acid intake during early pregnancy was recommended to avoid malformed embryos.

Furthermore, evaluation of the possible effect of 5-azacytidine and folic acid on the genomic DNA methylation pattern and the cytoplasmic protein content of the treated mice livers was investigated by means of DNA predigestion with methylation sensitive and non- sensitive restriction enzymes (HpaII and MspI), then amplifying the digested fragments using PCR with randomly selected primers and studying the effect on the gene expression using SDS-PAGE technique.

Results of agarose gel electrophoresis analysis of the predigested liver DNA showed similar pattern of fragments distribution between the DNA lanes of the (control and folic acid) liver samples. While, a different pattern of fragments distribution was recognized in the DNA lane of the 5-azacytidine liver sample.

Moreover, a different pattern of fragments distribution was identified in the lanes of DNA samples digested either with HpaII or MspI restriction enzymes for the DNA's of the (control and folic acid) liver samples. Whereas, a similar distribution pattern was recognized in the DNA lane of the 5-azacytidine liver sample.

On the other hand, results of RAPD-PCR amplification of the predigested DNA using the primer OPE-7 showed a similar pattern of fragment distribution in DNA lanes of the (control and folic acid) liver samples. While, a polymorphism was recognized in the DNA lanes of the 5-azacytidine liver sample.

Furthermore, a possible effect on the cytoplasmic proteins of mice liver might be detected using the sodium dodecylsulphate - polyacrylamide gel electrophoresis, where the protein sample of the 5-azacytidine treated liver showed a polymorphism in the calculated (Rm- value)of the migrating proteins.Whereas, similar values were calculated for the proteins of the control and folic acid samples.

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الإهـــداء

إلى من علمتني معنى الايمان
جدتي رحمها الله

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إلى بلـــدي

أهدي جهدي المتواضع هذا.

أسل
٢٢-٠٧-٢٠٠٥

بسم الله الرحمن الرحيم

اللَّهُ لا إِلَهَ إِلَّا هُوَ الْحَيُّ الْقَيُّومُ لا تَأْخُذُهُ سِنَةٌ وَلا نَوْمٌ لَهُ مَا فِي السَّمَاوَاتِ وَمَا فِي الْأَرْضِ مَنْ ذَا الَّذِي يَشْفَعُ عِنْدَهُ إِلَّا بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلا يُحِيطُونَ بِشَيْءٍ مِنْ عِلْمِهِ إِلَّا بِمَا شَاءَ وَسِعَ كُرْسِيُّهُ السَّمَاوَاتِ وَالْأَرْضَ وَلا يَؤُدُهُ حِفْظُهُمَا وَهُوَ الْعَلِيُّ الْعَظِيمُ

صدق الله العظيم
(البقرة: ٢٥٥)

الخلاصـــــة

تضمنت هذه الدراسة استخدام (٥٠٠) فأرة بيضاء من سلالة *Swiss Albino* والتي تراوحت أعمارها بين (٨-١٢) أسبوعاً وأوزانها بين (٢٣- ٢٥) غراما حيث شملت الدراسة أربعة أجزاء:

- ❖ تم في الجزء الأول تحديد الدورة النزوية لإناث فئران مجاميع التزاوج و التي استخدمت للحصول على الأجنة المستخدمة في إنتاج انسجه الفايبروبلاست و حيث تم استخدام أجنة بأعمار مختلفة (٩ و١١ و١٣) يوم والتي أظهرت نتائج الفحوصات المظهرية فيما بعد أن الأجنة بعمر (١١) يوم هي الأنسب للحصول على مزارع فايبروبلاست ناجحة.

- ❖ أما في الجزء الثاني من هذا البحث فقد تمت دراسة التأثير البايولوجي لثلاث أوساط زرعية مختلفة في دعم نمو نوعين من الخلايا الطبيعية (الفايبروبلاست) والسرطانية (البلازماسايتوما) حيث أظهرت الدراسات المظهرية وقياسات معدلات النمو أن وسط (RPMI 1640) هو الأفضل لنمو هذين النوعين من الخلايا.

- ❖ أما بالنسبة للتأثيرات خارج الجسم للازاسايتدين على نمو وتشكل خلايا الفايبروبلاست والبلازما سايتوما فقد تم بحثها في الجزء الثالث من هذه الدراسة حيث أثبتت نتائج الفحوصات وجود تأثيرات سمية لهذا المركب على نمو الخلايا من كلا النوعين أما بالنسبة لتشكل الخلايا فقد لوحظ تأثر خلايا الفايبروبلاست بالمعاملة حيث ظهرت بشكل مفرد خلايا مختلفة الأحجام أما بالنسبة لخلايا البلازما سايتوما فقد أدت المعادلة إلى نشوء خلايا مقاومة لوسط (HAT) والمسماة بخلايا (HPRT + ve) والتي اعتمدت إعدادها على تركيز الازاسايتدين المستخدم.

- ❖ في الجزء الرابع من هذه الدراسة، تم دراسة التأثيرات الداخلية لمركب الازاسايتدين وحامض الفوليك على: وزن الجسم والأعضاء التناسلية الذكرية ونشوء الأجنة وكروموسومات نخاع العظم والتعبير الجيني على مستوى الدنا والبروتين المستخدم في خلايا الكبد.

- ❖ أظهرت النتائج أن معاملة الفئران بالازاسايتدين أدى إلى انخفاض معنوي عالي في وزن الجسم وتأثيرات مختلفة على النبيبيبات المنوية (تشوهات في النبيب وانسلاخ في الخلايا غير الناضجة ونشوء خلايا عملاقة) و تشوهات في وظائف الحيامن من ناحية الحركة، الحيوية

والتركيز أدى إلى تراجع في وظائف الحيامن وانخفاض معدلات الخصوبة في الذكور المعاملة وزيادة نسبة الأجنة المفقودة قبل وبعد الانغراس عند الإناث الحوامل المعاملة.

- أما بالنسبة لمعاملة الحيوانات بحامض الفوليك فقد أثبتت النتائج عدم وجود أي تأثير لهذا الحامض على أعضاء الفئران المختلفة.

- من ناحية أخرى فقد لوحظ أن معاملة الإناث الحوامل بحامض الفوليك قبل المعاملة الحرارية أدى إلى تقليل نسبة الخسائر في الأجنة وتقليل نسبة التشوهات.

- من ناحية أخرى فان دراسة تأثيرات المعاملة بالازايتدين وحامض الفوليك على الدنا والبروتين في كبد الفئران المعاملة فقد تمت دراسته بواسطة أنزيمات التقييد HpaΠ وMspI وتقنية RAPD-PCR وتقنية SDS-PAGE حيث أظهرت النتائج اختلافاً في توزيع قطع الدنا بين نماذج الكبد المعاملة بحامض الفوليك والازايتدين وبين نماذج السيطرة السالبة للدنا المعامل بالأنزيمات وغير المعامل بها.

- من ناحية أخرى فقد لوحظ اختلاف أيضاً في نمط تقطيع الأنزيمات HpaΠ وMspI بين نماذج السيطرة والمعاملة بحامض الفوليك والازايتدين .

- علاوة على ذلك فان احتمالية تأثير هذه المعاملات على بروتينات الكبد تمت دراسته بواسطة تقنية SDS-PAGE حيث تم ملاحظة وجود اختلاف في قيمة(Rm) المحسوبة في ترحيل بروتينات نماذج الكبد للفئران المعاملة بحامض الفوليك و الازايتدين.

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Developmental Effects of 5-azacytidine and Folic acid In mice

A thesis

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In a partial fulfillment of the requirements

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Of science in Biotechnology

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التأثيرات التشكلية للأزاسايتدين وحامض الفوليك في الفئران

دراسة مقدمة الى كلية العلوم في جامعة النهرين

كجزء من متطلبات نيل درجة دكتوراه فلسفة في علم التقنيات الأحيائية

مقدمة من قبل

أسل عزيز توفيق القصاب

بكالوريوس تقانة أحيائية ١٩٩٨

ماجستير تقانة أحيائية ٢٠٠٠

جمادى الثانى ١٤٢٦

تموز ٢٠٠٥

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Doctor in philosophy of Science in Biotechnology.

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Scientific Degree:
Date:
Signature:
(Chairman)

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Supervisor Certification

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