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A Review on the Genotoxic Effects of Some Synthetic Progestins

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Abstract: The present review gives the details of the genotoxic studies carried out till date for some selected synthetic progestins. Mutagenicity is defined as a permanent change in content or structure of the genetic material of an organism. A mutagenic hazard can be manifested as a heritable change resulting from germ-line mutations and/or somatic mutations leading to cancer or other chronic degenerative processes such as aging. Reactive Oxygen Species (ROS) generated through normal metabolic processes or from toxic products, can lead to a state of oxidative stress that contributes to the pathogenesis of a number of human disease by damaging lipids, protein and DNA. Oral contraceptives have been used since the early 1960s and are now used by about 90 million women world wide. The pill is given as a combination of an estrogen and a progestogen. The estrogen component of combined oral contraceptives is either ethinylestradiol or mestranol and the progestogens used are cyproterone acetate, desogestrol, ethynodiol diacetate, levonorgestrel, lynestrenol, megestrol acetate, norethisterone, norethisterone acetate, norethynodrel, norgestimate and norgestrel. Little is known about the long term health risks and potential protective effects of these individual components. Synthetic progestins induced the genotoxic damage and also various types of cancers, both singly as well as in combination with estrogens. Various synthetic progestins have been tested for their genotoxic effects in different experimental models, using different genotoxic end points. Ethynodioldiacetate, norethynodrel, norgestrel, lynestrenol and medroxyprogesterone acetate were found to be genotoxic only in the presence of metabolic activation supplemented with NADP. Megestrol acetate, cyproterone acetate and chlormadinone acetate were found to be genotoxic in the absence of metabolic activation. On the basis of reports available it is suggested that the progestins in which double bond between carbon-6 and carbon-7 is present, they undergo nucleophilic reaction and generates free radical in the system to show the genotoxic effects and the progestins in which double bond between carbon-6 and carbon-7 is absent, they need metabolic activation like estrogens, such as estradiol-17β and ethinylestradiol to show the genotoxic effects.

Key words: Synthetic progestins, genotoxicity, reactive oxygen species, structural relationship, oral contraceptives

INTRODUCTION

Steroid hormones are the members of lipid compounds. They are mainly secreted by adrenal cortex of mammals, testis in male, ovary in the female and the placenta of mammals. Steroids consist of a tetracyclic nucleus, which is named cyclopentanoperhydro-phenanthrene (Fig. 1).

The phenanthrene portion of the nucleus is comprised of rings A, B and C, while D is the cyclopentane portion. Steroid hormones are responsible for a number of physiological and pharmacological effects in humans and other mammals. There are four basic classes of steroid hormones (Gorbman *et al.*, 1983).
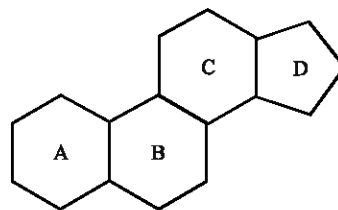

Fig. 1: Steroid nucleus

Adenocorticoids: Adrenocorticoids are grouped into the (i) Glucocorticoids: Cortisol, corticosterone and cortisone. They facilitate the formation of carbohydrates from non-carbohydrate sources (gluconeogenesis) and (ii)

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Mineralocorticoids: They have ability to affect water and electrolyte metabolism (favoring retention of Na^+ and excretion of K^+), e.g., Aldosterone.

Estrogens: Estrogens have the ability (secreted by the ovarian follicle) to stimulate female secondary sex characteristics and to help maintain the female reproductive tract e.g., estrone, estradiol and estriol.

Progestins: They stimulate the uterus and maintain uterine development during pregnancy (secreted by the corpus luteum of the ovary) e.g., Progesterone, 17α-hydroxyprogesterone.

Androgens: They stimulate male characteristics and maintain male sex accessory glands and ducts (secreted by testis) e.g., testosterone, 5 α-dihydrotestosterone and androstenedione. The usefulness of above classification scheme is limited to some mammalian species, because glucocorticoids stimulates aspects of female reproduction on in bony fishes. Aldosterone is usually classified as a mineralocorticoid, but it is a potent glucocorticoid in some animals.

Structure and nomenclature of steroids: The basic nucleus of steroids has 17 carbon atoms as shown in the Fig. 2.

The nucleus has six asymmetric carbon atoms, shown with asterisk in the Fig. 2. The nucleus is a flat structure lying in the plane of the page. If the hydrogen projects towards the observer, it is said to be in the cis or-β position (solid line) and if the hydrogen project away from the observer, it is said to be in the trans or α-position (broken lines) as shown below:
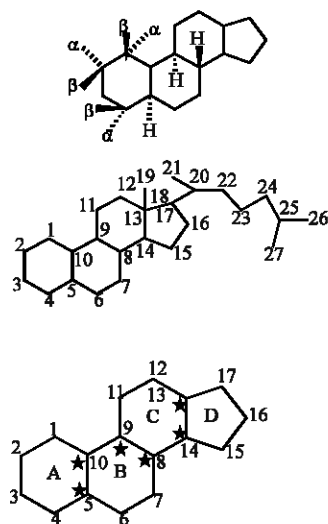

Fig. 2: Structure of steroids

Additional carbons can be attached to the 17-carbon nucleus to form other classes of steroid compounds. If a methyl atom is attached to carbon 13, a new compound known as estrone (C-18) is formed. If a second methyl group is added to carbon 10, androstane (C-19) is formed.

Metabolism of steroids: Ovary, testis, adrenal gland and placenta have the ability to produce steroidal hormones, which is favoured by the trophic hormones such as andrenocorticotrophic Hormone (ACTH), Luetinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Progesterone Releasing Hormone (PRL) and its receptor in the target tissues. It is also possible that one steroid secreting organ can take up steroid originally secreted by another organ and modified its activity. For example, mammalian adrenal cortex can take up progesterone secreted by the ovary and modifies it to corticoids. Chemical modifications may also occur in the brain, skin, salivary glands and other tissues. Steroid hormones are synthesized from a C_{27} precursor steroid, cholesterol. There are multiple sources of this steroid hormone precursor: *de novo* synthesis within the endocrine organ and uptake from the circulating pool that is maintained by the liver or supplied in the diet. In the blood cholesterol circulates in the form of cholesterol esters bound to Low and High Density Lipoprotein (LDL and HDL). For the synthesis of the steroid hormone the breaking off of the six carbon side chain of the cholesterol occurs in mitochondria (Gorbman *et al.*, 1983).

The inner membrane of the mitochondria contains an oxygenases known as cytochrome P450, which breaks the side chain of the cholesterol and results in the formation of pregnenolone (C_{21}). Pregnenolone is further transported to endoplasmic reticulum, where it is either oxidized to form progesterone or hydroxylated to form 17-hydroxypregnenolone. Further with the help of hydroxylating enzyme, desmolase and various cofactors triphosphopyridine nucleotide (TPNH) and molecular oxygen give rise to various steroid hormones (Fig. 3). The catabolism of steroid occurs in both steroidogenic and peripheral tissues. The major site of catabolism is the liver although the kidney and other tissues may catabolize circulating steroids to some extent. The major catabolic enzymes are involved in reductive reactions, particularly in the ring A and carbons 3 and 20. They are reduced to more water soluble and in active steroid sulfates and glucoronides that are eliminated through the urine or through the bile fluid, with ultimate excretion in the feaces (Gorbman *et al.*, 1983).
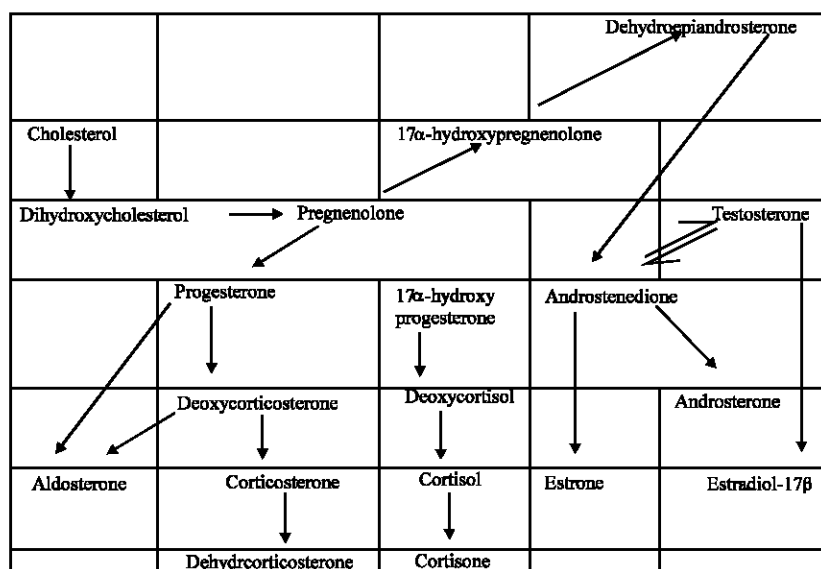
Fig. 3: Biogenesis of steroid hormones

Action of Birth Control Pills (BCPs): Normally the pituitary glands produces two hormones called Follicle Stimulating Hormone (FSH) and Leutenizing Hormone (LH). These hormones serve to stimulate the ovary to produce an egg at each menstrual cycle (to ovulate). The ovary is also the production site for the woman's two central female hormones estradiol and progesterone, (Progestin) (Elstein *et al.*, 1976). Birth Control Pills (BCPs) are a combination of synthetic estrogen and progestin. BCPs fool the pituitary gland so that it produces less FSH and LH. By reducing the FSH and LH required for ovulation, birth control pills suppress, but do not eliminate ovulation. BPCs may have two main effects (Wolf *et al.*, 1979; Chang and Hunt, 1970): (i) They thin the inner lining of the uterus (called the endometrium) depleting it of glycogen and blood supply, (ii) BCPs may thicken the cervical mucus, making it more difficult for the sperm to travel up through the cervix. Intergrins are a group of adhesion molecules that have been implicated as playing an important role in fertilization and implantation. According to Somkuti *et al.* (1996) there are several types of integrins and it is believed that the endometrium is most receptive to implantation when it expresses certain types of integrins. BPCs change the type of integrins that the endometrial lining produces and makes the implantation difficult of the pre-born child (Somkuti *et al.*, 1996).

Genotoxic damage and cancer risks of oral contraceptives: The type of oral contraceptives prescribed differs between countries and both the type of oral contraceptive and the doses of estrogens and progestogens have changed between and within

countries overtime (Joosten *et al.*, 2004). Chromosomal abnormalities and sister chromatid exchanges have been reported in peripheral blood lymphocytes of women taking oral contraceptives (Carr, 1967, 1970; Goh, 1967; Murthy and Prema, 1979). There is sufficient data which indicates that the prolonged use of the synthetic progestins can cause cancer among animals and humans (Rudali, 1975; El-Etreby and Gräf, 1979; Misdorp, 1991; Deml *et al.*, 1993; Martelli *et al.*, 1996; Heinemann *et al.*, 1997; Maier and Herman, 2001).

SYNTHETIC PROGESTINS

Synthetic progestins have a wide spread use in medicine but their side effects are often debatable. Due to the genotoxic hazards and carcinogenic risks of synthetic progestins it is important to review the studies carried out till date. The present review is limited to some commonly use synthetic progestins in oral contraceptives formulations. Progestins were first isolated in 1993 and progesterone itself was synthesized in the 1940s. There are different classes of progestins, such as progesterone, retroprogesterone, progesterone derivative, 17α-hydroxy progesterone derivatives (pregnanes), 17α-hydroxy nor progesterone derivatives (non-pregnanes), 19-norprogesterone derivatives (nor pregnanes), 19-nortestosterone derivatives (estranes), 19-nortestosterone derivatives (gonanes) and spirolactone derivative. Schindler *et al.* (2003) has classified progestins in nine groups (Table 1) and the details for the genotoxic studies carried out till date is given in Table 2.

Table 1: Types of Progestins

No.	Progestin	Example
1.	Progesterone	Natural progesterone
2.	Retroprogesterone	Dydrogesterone
3.	Progesterone derivative	Medrogestone
4.	17α-Hydroxyprogesterone derivatives (Pregnanes)	Medroxy progesterone, Megestrol acetate, Chlormadinone acetate, Cyproterone acetate
5.	17α Hydroxynorprogesterone derivatives (Norpregnanes)	Gestonorone Caproate, Nomegestrol Acetate
6.	19-Norprogesterone derivative (Nor pregnanes)	Demegestone, Promegestone, Nesterone, Trimegestone
7.	19-Nortestosterone derivatives) (Estranes)	Norethisterone, Norethisterone Acetate, Lynestrenol, Ethynodiol Diacetate, Norethynodrel
8.	19-Nortestosterone derivatives (Gonanes)	Norgestrel, Levonorgestrel, Desogrestred, Etenogestrel, Gestodene, Norgestimate, Dienogest
9.	Spirolactone derivative	Drospirenone

Table 2: Genotoxicity studies for synthetic progestins carried out till date

No.	Synthetic progestins	Test models	End-points	Conclusions	References
1	Medroxy progesterone acetate	Ames Test	Gene mutation	Negative	Zeiger *et al.* (1992) and Herzog and Leuscher (1995)
		V79 *in vitro*	Gene mutation	Negative	Herzog and Leuschner (1995)
		Rat hepatocytes *in vitro*	UDS	Negative/Positive	Martelli *et al.* (2003)
		Human hepatocytes *in vitro*	UDS	Negative	Martelli *et al.* (2003)
		Mouse BM *in vivo*	MN	Negative	Morita *et al.* (1997)
		Human PBL *in vitro*	CA	Negative	Herzog and Leuschner (1995)
	Human PBL *in vitro*	Human PBL *in vitro*	CA	Positive	Siddique *et al.* (2006a)
		Human PBL *in vitro*	SCE	Positive	Siddique *et al.* (2006a)
		Rat BM *in vivo*	CA	Negative	Herozog and Leuschner (1995)
2	Chlormadinone acetate	Ames Test	Gene mutation	Negative	Joosten *et al.* (2004)
		Rat liver *in vivo*	32P	Positive	Feser *et al.* (1996) and Topinka *et al.* (1995)
		Rat hepatocytes *in vitro*	UDS	Negative	Topinka *et al.* (1995)
		Rat hepatocytes *in vitro*	32P	Positive	Topinka *et al.* (1995)
		Human hepatocytes *in vitro*	32P	Positive	Werner *et al.* (1997)
		Rat liver *in vivo*	MN	Positive	Martelli *et al.* (1996)
		Human PBL *in vitro*	CA	Negative	Stenchever *et al.* (1969)
		Human PBL *in vitro*	CA	Positive	Siddique and Afzal (2004b); Siddique and Afzal, (2005a); Siddique *et al.* (2008a)
		Human PBL *in vitro*	SCE	Positive	Siddique and Afzal (2004b); Siddique and Afzal, (2005a); Siddique *et al.* (2008a)
		Mouse BM *in vivo*	CA	Positive	Siddique and Afzal (2004a)
			SCE	Positive	Siddique and Afzal (2004a)
3	Ethynodiol diacetate	Human PBL *in vitro*	CA	Positive	Siddique and Afzal (2004c); Siddique *et al.* (2007a)
			SCE	Positive	Siddique and Afzal (2004c); Siddique *et al.* (2007a)
4	Megestrol acetate	Rat liver *in vivo*	32P	Positive	Feser *et al.* (1996) Topinka *et al.* (1995)
		Rat hepatocytes *in vitro*	UDS	Negative	Topinka *et al.* (1995)
		Rat hepatocytes *in vitro*	32P	Positive	Topinka *et al.* (1995)
		Human hepatocytes *in vitro*	32P	Positive	Werner *et al.* (1997)
		Human PBLs *in vitro*	CA	Negative	Stenchever *et al.* (1969)
		Rat liver *in vivo*	MN	Positive	Martelli *et al.* (1996)
		Mouse BM *in vivo*	SCE	Positive	Siddique *et al.* (2005a)
			CA	Positive	Siddique *et al.* (2005a)
5	Cyproterone acetate	V79 *in vitro*	Gene mutation	Negative	Kasper *et al.* (1995)
		V79 *in vitro*	Gene Mutation	Negative	Lang and Reimann (1993)
		Big Blue rat	Gene mutation	Positive	Krebs *et al.* (1998)
		Ames Test	Gene mutation	Negative	Lang and Reimann (1993)
		Rat hepatocytes *in vitro*	32P	Positive	Topinka *et al.* (1995) Topinka *et al.* (1995)
		Rat hepatocytes *in vitro*	UDS	Positive	Topinka *et al.* (1995) Neumann *et al.* (1992) Kasper *et al.* (1995)
		Rat hepatocytes *in vitro*	UDS/SSB	Positive/Negative	Martelli *et al.* (1995)
		Human hepatocytes *in vitro*	UDS	Positive	Martelli *et al.* (1995)
		Human hepatocytes *in vitro*	32P	Positive	Werner *et al.* (1997)
		Human hepatocytes *in vitro*	SSB	Positive	Martelli *et al.* (1995)
		Rat liver *in vivo*	UDS	Positive	Kasper and Mueller (1996)
		Rat liver *in vivo*	32P	Positive	Feser *et al.* (1996)
		Rat liver *in vivo*	MN	Positive	Martelli *et al.* (1996)
		V79 *in vitro*	CA	Negative	Kasper *et al.* (1995)
		Rat hepatocytes *in vitro*	MN	Positive	Kasper *et al.* (1995)
		Human PBLs *in vitro*	CA	Positive	Siddique and Afzal (2005b); Siddique and Afzal, 2004d; Siddique *et al.*, 2006b, Siddique *et al.*,

Table 2: Continued

				2007b; Siddique *et al.*, 2008b;
	Human PBLs *in vitro*	CA	Negative	Reimann *et al.* (1996)
	Human PBLs *in vitro*	SCE	Positive	Siddique and Afzal (2005b); Siddique and Afzal, 2004d; Siddique *et al.* (2006b); Siddique *et al.* (2007b); Siddique *et al.* (2008b);
	Mouse BM *in vivo*	MN	Negative	Reimann *et al.* (1996)
(6) Lynestrenol	Human PBLs *in vitro*	CA	Positive	Siddique and Afzal (2005e)
	Human PBLs *in vitro*	SCE	Positive	Siddique and Afzal (2005e)
	Mouse BM *in vivo*	CA	Positive	Siddique and Afzal (2005c)
	Mouse BM *in vivo*	SCE	Positive	Siddique and Afzal (2005c)
(7) Norgestrel	Human PBLs *in vitro*	SCE	Positive	Ahmad *et al.* (2001)
	Human PBLs *in vitro*	SCE	Positive	Siddique *et al.* (2006c); Siddique *et al.* (2007c)
	Human PBLs *in vitro*	CA	Positive	Ahmad *et al.* (2001)
	Human PBLs *in vitro*	CA	Positive	Siddique *et al.* (2006c); Siddique *et al.* (2007c)
	Mouse BM *in vivo*	MN	Negative	Jordan (2002)
(8) Norethisterone	Ames test	Gene mutation	Negative	Lang and Reimann (1993); Dhillon and Dhillon (1996)
	Mouse HMA	Gene mutation	Negative	Dhillon and Dhillon (1996)
	Rat liver *in vivo*	32P	Negative	Feser *et al.* (1996)
	Human PBLs *in vitro*	SCE	Positive	Dhillon and Dhillon (1996)
	Human PBLs *in vitro*	SCE	Negative	Ahmad *et al.* (2001)
	Mouse BM *in vivo*	SCE	Positive	Dhillon and Dhillon (1996)
	Rat hepatocytes *in vitro*	UDS	Positive/Negative	Martelli *et al.* (2003)
	Human hepatocytes *in vitro*	UDS	Positive/Negative	Martelli *et al.* (2003)
	Male rat hepatocytes *in vitro*	UDS	Positive	Joosten *et al.* (2004)
	Human PBLs *in vitro*	CA	Positive	Dhillon and Dhillon (1996)
	Human PBLs *in vitro*	CA	Negative	Stenchever *et al.* (1969)
	Human PBLs *in vitro*	CA	Negative	Ahmad *et al.* (2001)
	Human PBLs *in vitro*	SCE	Negative	Ahmad *et al.* (2001)
	Mouse BM *in vivo*	MN	Positive	Dhillon and Dhillon (1996)
	Rat liver *in vivo*	MN	Positive/Negative	Martelli *et al.* (1998)
(9) Norethynodrel	Ames Test	Gene mutation	Negative	Lang and Reimann (1993)
	Male rat hepatocytes *in vitro*	UDS	Positive	Joosten *et al.* (2004)
	Rat hepatocytes *in vitro*	UDS	Negative	Joosten *et al.* (2004)
	Human PBLs *in vitro*	SCE	Positive	Siddique and Afzal (2005d); Siddique *et al.* (2006d); Siddique *et al.* (2007d)
	Human PBLs *in vitro*	CA	Positive	Siddique and Afzal (2005d); Siddique *et al.* (2006d); Siddique *et al.* (2007d)
	Mouse bone marrow cells	CA	Positive	Siddique and Afzal (2003)
	Mouse bone marrow cells	SCE	Positive	Siddique and Afzal (2003)
(10) Dimethisterone	Human PBL *in vitro*	CA	Negative	Stenchever *et al.* (1969)
(11) Progesterone	L5178Y *in vitro*	Gene mutation	Positive/Negative	Myhr and Caspary (1988)
	Rat liver *in vivo*	32P	Negative	Feser *et al.* (1996)
	Male rat hepatocytes *in vitro*	UDS	Negative	Oshiro *et al.* (1986)
	Rat hepatocytes *in vitro*	UDS	Positive/Negative	Martelli *et al.* (2003)
	Human hepatocytes *in vitro*	UDS	Negative	Martelli *et al.* (2003)
	Human hepatocytes *in vitro*	32P	Negative	Werner *et al.* (1997)
	Human PBLS *in vitro*	CA	Negative	Stenchever *et al.* (1969)
	Rat liver *in vivo*	MN	Positive	Martelli *et al.* (1996)
	Chinese Hamster DON cells *in vitro*	Aneuploidy	Negative	Wheeler *et al.* (1986)

Medroxy progesterone acetate (CAS: 71-58-9)

(16)-17-(Acetyloxy)-6-methylpregn-4-ene-3,20-dione)

Colour: White or off-white

Odour: Odourless

Solubility: Medroxyprogesterone acetate is practically insoluble in water, sparingly soluble in alcohol and in methyl alcohol, slightly soluble in ether freely soluble in chloroform, acetate, dioxin, dimethylsulphoxide.

Fig. 4: Medroxy progesterone acetate

Postulated mode of action: Medroxy progesterone acetate (Fig. 4) is a progestin that is derived from the naturally occur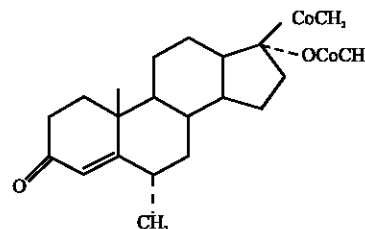ring female hormone, progesterone. It is the derivative of 17α-hydroxyprogesterone (Pregnanes). It is used to treat abnormal uterine bleeding, promote menstrual cycles and to treat symptoms of the

menopause. Medroxyprogesterone is use to promote menstruation when women do not begin naturally to menstruate at puberty (called primary amenorrhea) or if they stop menstruating before menopause (called secondary amenorrhea). It is used in the treatment of endometrial, prostate and renal cancer. It is also used for treating abnormal bleeding from the uterus in many situations. It is also used in combinations with estrogens for treating symptoms of menopause in order to prevent unchecked growth of the endometrium that may lead to endometrial cancer. It is secreted in breast milk, but the effect on infant has not been determined (Gilstrap and Little, 1992; Briggs *et al.*, 2005). After oral intake medroxyprogesterone does not undergo any first pass effect. The bioavailability is 100%. It has no binding affinity to Sex-Hormone Binding Globulin (SHGB) and Corticosteroid Binding Globulin (CBG) and in serum medroxy progesterone acetate is bound to albumin for 88%. It is extensively metabolized in the liver (Schindler *et al.*, 2003).

Cancer studies: Medroxyprogesterone acetate causes reversible changes in the endometrium, from proliferative to secretary or suppressed. Medroxyprogesterone acetate induced adeno carcinomas of the mammary gland in female mice and malignant mammary tumors in dogs. In female dogs a dose-related increase in the incidence of large mammary nodules was also found after intra-muscular administration (Lanari *et al.*, 2001).

Genotoxic studies: Medroxyprogesterone acetate was reported to be negative in Ames test (Zeiger *et al.*, 1992; Lang and Reimann, 1993) and V79 cells *in vitro* (Herzog and Leuschner, 1995). It was reported positive for unscheduled DNA synthesis in rat hepatocytes *in vivo* (Martelli *et al.*, 2003), but negative in cultured human hepatocytes (Martelli *et al.*, 2003). It does not induced micronucleus in mouse bone marrow cells (Morita *et al.*, 1997) and chromosomal aberrations in rat bone marrow cells (Herzog and Leuschner, 1995). It did not form DNA adducts in human lymphocytes (Werner *et al.*, 1997) and was reported to form DNA adducts in human liver slices *in vitro* (Feser *et al.*, 1998). It induced chromosomal aberrations and sister chromatid exchanges in cultured human peripheral blood lymphocytes only in the presence of metabolic activation supplemented with NADP (Siddique *et al.*, 2006a).

Chlormadinone acetate (CAS No. 302-22-7)
(6-chloro-17-hydroxypregna-4,6-diene-3, 20-dione acetate)
Colour: White or Creamy white
Odour: Odourless

Solubility: Chlormadinone acetate soluble in water, sparingly soluble in alcohol, soluble in acetone, ether and dimethysulphoxide.

Postulated mode of action: Chlormadinone acetate (Fig. 5) is a synthetic progesterone analogue. It is a derivative of 17α-hydroxyprogesterone (pregnanes), having chlorine atom at carbon-6 (Schindler *et al.*, 2003). Chlormadinone acetate, with its anti-androgenic action (direct inhibitory effect on prostrate), exerts an inhibitory effect on hypertrophic prostrate, atrophic effects on prostrate. PROSTAL®-l Tablets contain 50 mg of chlormadinone acetate. When one tablet of PROSTAL-L Tablets was orally administrated to healthy male adults at the fasting state, the plasma concentration reached the maximum level at 5.1 h (T_{max}) after the administration with a half life of 10.2 h ($T_{1/2}$) showing the sustained release pattern of plasma concentration compared with ordinary chlormadinone acetate tablets. After oral intake chlormadinone acetate is rapidly absorbed and undergoes nearly no first pass metabolism. Therefore, the bioavailability is nearly 100%. It has no binding affinity to Estrogen Receptor (ER), Mineralocorticoid Receptor (MR) sex-hormone binding receptor and Corticosteroid-Binding Globulin (CBG). Chlormadinone acetate elimination occurs slowly. After 7 days only 34% of the dose is extracted. The reduction of the 3-keto-group results in the inactivation. 3-hydroxy-chlormadinone acetate is the important metabolite, which shows 70% of the anti-androgenic activity. Hydroxylation occurs at positions C2α, C3β and C15β. The majority of the metabolites are excreted renally, predominantly as glucoronides (Schindler *et al.*, 2003). In cattle Chlormadinone is used for oestrus synchronizations at daily oral doses of 12 mg per animal for upto 20 days. The minimal effective level in human was 50 μg day^{-1} (CVMP, 200). Chlormadinone acetate is also used in sheep and goats for the same indication at daily oral doses of 2.5 mg per animal and in horses at daily oral doses of 12 mg per animal for upto 20 days. Its oral LD50 is 6400 mg kg^{-1} b.wt. but intraperitoneal LD50 in mice is 90 mg kg^{-1} b.wt. (Siddique and Afzal, 2004a).
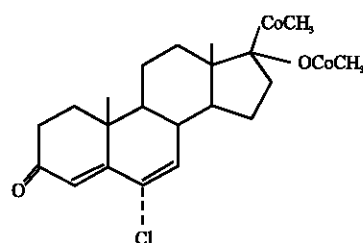

Fig. 5: Chlormadinone acetate

Cancer studies: Chlormadinone produced mammary tumors in dogs and also increase the incidence of mammary gland hyperplasia and mammary nodules (El Etreby and Graf, 1979). There is no data available on the genetic and related effects of chlormadinone acetate alone in humans.

Genotoxic studies: It was found negative in bacterial test system, unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* (Topinka *et al.*, 1995) and chromosomal aberrations in human peripheral blood lymphocytes *in vitro* (Stenchever *et al.*, 1969), but in other study was reported to induced chromosomal aberrations and sister chromatid exchanges in cultured human lymphocytes (Siddique and Afzal, 2004b, 2005a; Siddique *et al.*, 2008a). It was reported to form DNA adducts in rat liver *in vitro* (Topinka *et al.*, 1995; Feser *et al.*, 1996; Brambilla and Martelli, 2002) and human hepatocytes *in vitro* (Topinka *et al.*, 1995; Werner *et al.*, 1997) and micronucleus in rat liver cells *in vivo* (Martelli *et al.*, 1996).

Ethynodiol diacetate CAS No. (297-76-7)
Colour: White or almost white
Odour: Odourless or almost odourless

Solubility: It is practically insoluble in water, soluble in alcohol, chloroform, ether and dimethyl sulphoxide. It is sparingly soluble in fixed oils.

Postulated mode of action: Ethynodiol diacetate (Fig. 6) is a derivative of 19-nortestosterone (estranes). It is used in the treatment of hypermenorrhea (menorrhagia), pain associated with endometriosis, dysmenorrhoea and dysfunctional uterine bleeding. It is also used as a female contraceptive. Ethynodioldiacetate tricks the body processes into thinking that ovulation has already occurred, by maintaining high levels of the synthetic progesterone. This prevents the release of eggs from ovaries. Ethynodiol diacetate and ethinylestradiol combination is used in oral contraceptives i.e., Demulen® and Zovia®. This combination prevents pregnancy by
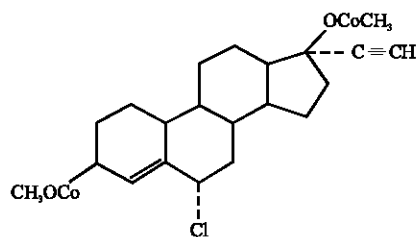

Fig. 6: Ethynodiol diacetate

preventing ovulation (egg release). It causes a variety of hormonal changes. To prevent pregnancy after intercourse, the medicine either prevents or delays ovulation (egg release). Ethynodiol diacetate binds to the progesterone and estrogen receptors target cells include the female reproductive tract, mammary gland, the hypothalamus and the pituitary. Once bound the receptor, it shows the reduction in the frequency of release of Gonadotropin Releasing Hormone (GnRH) from the hypothalamus and blunt preovlatory LH (Lutenizing hormone) Surge. It is metabolized in liver or gut wall, to norethisterone and to sulphate and glucoronide conjugates. Its half life is about 25 h.

Cancer studies: Ethynodioldiacetate increased the incidence of benign liver tumours in male mice and of mammary tumours in castrated male mice. There is no data available on the genetic and related effects of ethynodiol diacetate alone in humans. Oral administration of ethynodiol diacetate; in combination with mestranol to mice, increased the incidence of pituitary tumours. Ethynodiol diacetate plus ethinylestradiol increased the incidence of pituitary tumours and of malignant tumours of connective tissues of the uterus in mice. In rats it produced mammary tumours.

Genotoxic studies: Ethynodiol diacetate was reported to induced chromosomal aberrations and sister chromatid exchanges on cultured human lymphocyte in the presence of metabolic activation supplemented with NADP (Siddique and Afzal, 2004c; Siddique *et al.*, 2007a).

Megestrol acetate (CAS: 595-33-5)
(17α-(Acetyloxy)-6-methyl-pregna-4,6-diene-3,20 dione)
Colour: White or creamy white
Odour: Odourless or almost odourless

Solubility: Megestrol acetate is practically in soluble in water, sparingly soluble in alcohol, very soluble in chloroform, slightly soluble in ether and in fixed oils.

Postulated mode of action: Megestrol acetate (Fig. 7) is a derivative of 17α-hydroxy progesterone (Pregnanes). It is used in oral contraceptives, breast and in the treatment of endometrial cancer. After oral intake the bioavailability of megestrol acetate is 100%. It is not bound to Sex Hormone Binding Protein (SHBG) or Corticosteroid Binding Globulin (CBG). It is bound to serum albumin. The most important metabolic pathways are hydroxylation reactions. It is metabolized by liver to glucoronide conjugates. It is excreted as conjugates metabolites via the urine and faeces (Schindler *et al.*, 2003).
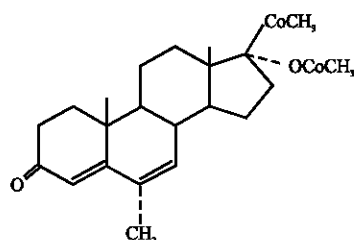
Fig. 7: Megestrol acetate

Fig. 8: Cyproterone acetate

Cancer studies: Megestrol acetate plus ethinylestradiol has also been tested for carcinogenicity by oral administration in mice and rats. In mice, an increased incidence of malignant mammary tumours was observed in both sexes, but no increase in incidence of tumours was seen in rats. There is no data available on the genetic and related effects of megesterol acetae alone in humans (Joosten *et al.*, 2004).

Genotoxic studies: It has been reported negative in unscheduled DNA synthesis test using rat hepatocytes; however, the presence of DNA adducts has been shown in rat liver *in vivo* and cultured human hepatocytes (Topinka *et al.*, 1995; Feser *et al.*, 1996; Werner *et al.*, 1997). It has also been shown to induce micronucleus in rat liver *in vivo*, but has failed to cause chromosomal aberrations in human peripheral blood lymphocytes *in vitro* (Stenchever *et al.*, 1969; Martelli *et al.*, 1996). It induced chromosomal aberrations and sister chromatid exchanges in mice bone marrow cells (Siddique *et al.*, 2005a).

Cyproterone acetate (CAS No: 427-51-0)
Colour: White or creamy white
Odour: Odourless or almost odourless

Solubility: Cyproterone acetate is practically insoluble in water, sparingly soluble in alcohol, very soluble in chloroform, acetone and dimethysulphoxide acetone, slightly soluble in ether and in fixed oils.

Postulated mode of action: Cyproterone acetate (Fig. 8) is a derivative of 17α-Hydroxyprogesterone (Pregnanes). In addition to the 6, 7 double bond, the 1,2 α-methyl group is present. Cyproterone acetate is a potent steroidal antiandrogen with progestational activity. It is used alone or in combination with ethinylestradiol or estradiol valerate in the treatment of women suffering from disorders associated with androgenization, e.g., acne or hisuitism. Cyproterone acetate competes with dihydrotestosterone for the androgen receptor and inhibits translocation of the hormone receptor complex in
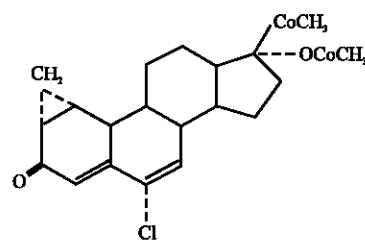
to the cell nucleus (Sciarra *et al.*, 1990). The bioavailablity is nearly 100%. Cyproterone acetate has no binding affinity to sex hormone binding globulin and corticosteroid binding globulin in the serum but 93% of compound is bound to serum albumin. It is stored in fat tissue and excreted slowly. The important metabolic steps are hydroxylation reaction and de-acetylation. The metabolite 15β hydroxycyproterone acetate shows only 10% of the progestogenic potency of cyproterone itself. The bio-activation of the cyproterone acetate involves the reduction of the keto group at carbon-3, which is followed by sulfonation of the hydroxy steroid. The resulting sulfoconjugate is supposed to be very unstable and can decompose to a reactive DNA binding carbonium ion (Schindler *et al.*, 2003).

Cancer studies: Cyproterone acetate is a tumour initiating agent in the liver of female rats (Deml *et al.*, 1993).

Genotoxic studies: Cyproterone acetate was found negative in V79 cells *in vitro* (Lang and Reimann, 1993; Kasper *et al.*, 1995) in Ames test (Lang and Reimann, 1993) and for micronucleus in mouse bone marrow cells *in vivo* (Reimann *et al.*, 1996; Martelli *et al.*, 1996) but it was positive for micronucleus in rat liver *in vivo*, for chromosomal aberrations in V79 cells (Kasper *et al.*, 1995) and the human peripheral blood lymphocytes *in vitro* (Reimann *et al.*, 1996). It induces mutation in Big Blue rat (Krebs *et al.*, 1998). It was also found positive for unscheduled DNA synthesis (UDS) test in rat hepatocytes (Neumann *et al.*, 1992; Kasper *et al.*, 1995; Topinka *et al.*, 1995; Martelli *et al.*, 1995), human hepatocytes *in vitro* and in rat liver *in vivo* (Kasper and Muller, 1996) In female rats, DNA adducts have been observed at low doses of cyproterone acetate, which are in the range of therapeutic doses used in women (Werner *et al.*, 1997). It was found to induce chromosomal aberrations and sister chromatid exchanges in human lymphocytes *in vitro* (Siddique and Afzal, 2005b, 2004d; Siddique *et al.*, 2006b, 2007b, 2008b).

Lynestrenol (CAS No 52-76-6) (17α)-19-Norpregn-4-en-20yn-17ol)
Colour: White
Odour: Odourless

Solubility: Lynestrenol is practically insoluble in water, sparingly soluble in alcohol, very soluble in acetone, chloroform, dimethysulphoxide.

Postulated mode of action: Lynestrenol (Fig. 9) is used as single cavity drug or in combination with estrogen, such as ethinylestradiol or mestranol in oral contraceptives (Siddique and Afzal, 2004e). It is the derivative of 19-nortestosterone. Lynestrenol is converted *in vivo* to norethisterone. It is metabolized by 3β-hydroxylation and dehydrogenation (Schindler *et al.*, 2003).

Cancer studies: The high doses of lynestrenol were associated with the increases incidence of mammary nodules and carcinomas (Misdorp, 1991). The LD50 value for lynestrenol for mice is 110 mg kg^{-1} body weight (Siddique and Afzal, 2005c).

Genotoxic studies: Lynestrenol induced chromosomal aberrations and sister chromatid ecology at the dosages of 13.75 and 27.50 mg kg^{-1} body weight in mice bone marrow cells (Siddique and Afzal, 2005c). It induced CA and SCE in cultured lymphocyte only in presence of metabolic activation (Siddique and Afzal, 2004e).

Norgestrel (CAS : 797-63-7)
(13β-Ethyl-17α-ethynyl-17β-hydroxygon-4en-3-one)
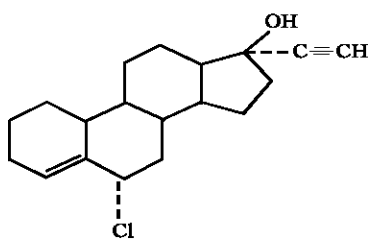**Colour:** White or almost white
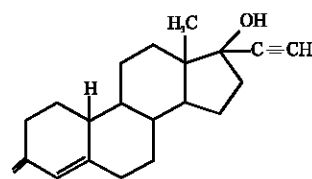**Odour:** Odourless

Fig. 9: Lynestrenol

Fig. 10: Norgestrel

Solubility: Norgestrel is practically insoluble in water, sparingly soluble in alcohol, methylene chloride and freely soluble in chloroform and dimethyl sulphoxide.

Postulated mode of action: Norgestrel (Fig. 10) is the derivative of 19-nortestosterone (gonanes). It is a combination of both active and inactive enantiomers of which only the levorotary form is biologically active. It is used as a oral contraceptive either as single agent or in combination with an estrogen. Norgestrel was approved by the FDA in 1973. Norgestrel after oral administration is readily absorbed from the gastrointestinal tract and is widely distributed in body fluids. Protein binding is > 90% and is primarily to Sex Steroid Binding Globulin (SSBG) and albumin. Its half life is 10.26 h. Metabolism is believed to be hepatic with an elimination half life of about 20 h. Elimination is mostly via the urine with minimal amounts excreted in the bile and milk. Approximately 0.1% of the daily dose passes into breast milk (Schindler *et al.*, 2003).

Cancer studies: It is reported to increase endometrial thickness during intrauterine progesterone therapy (Kresowik *et al.*, 2008).

Genotoxic studies: It was reported to be genotoxic in cultured human peripheral blood lymphocyte with and without metabolic activation (Ahmad *et al.*, 2001) and was negative for micronucleus in mouse bone marrow cells (Jordan, 2002). Norgestrel induced chromosomal aberration and sister chromatid exchanges in the presence of metabolic activation supplemented with NADP in culture human lymphocytes (Siddique *et al.*, 2006c, 2007c).

Norethisterone (CAS No : 68-22-4)
(17α)-17-hydroxy-19-norpregn-4en-2oyn-3-one)
Colour: White or yellowish white
Odour: Odourless

Solubility: Norethisterone is insoluble in water, sparingly soluble in alcohol and soluble in chloroform and dimethylsulphoxide.

Postulated mode of action: Norethisterone (Fig. 11) is also named norethindrone and is often used as norethisterone acetate (NETA). Both compounds are rapidly absorbed from the gastro intestinal tract. The bio availability is about 64; 36% are bound to sex hormone binding globulin; 61% to serum albumin and 3% are free in circulation. Its half life is 5-12 h and is metabolised in the intestinal wall and liver. The principal metabolite is 5-α-dihydronorethisterone (Schindler *et al.*, 2003).

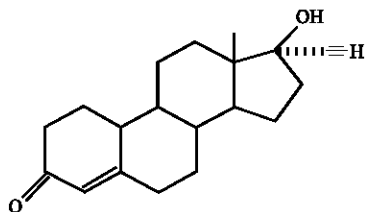Fig. 11: Northisterone

Fig. 12: Norethynodrel

Cancer studies: Norethisterone and its acetate were tested by oral administration in mice and rats and by subcutaneous implantation in mice. In mice, norethisterone and its acetate increased the incidence of benign liver cell tumours in males. It increased the incidence of pituitary tumours in females and produced granulosa cell tumours in the ovaries of females. It also increased the incidence of benign liver cell tumours and benign and malignant mammary tumours in male rats (Ahmad *et al.*, 2001).

Genotoxic studies: Northisterone has been found to be negative in bacterial tests (Lang and Reimann, 1993; Dhillon and Dhillon, 1996). The widely used contraceptive steroid has been studied with respect to SCE and UDS induction and DNA adducts formation, in a range of *in vitro* and *in vivo* systems. Induction of UDS in human and rat hepatocytes was either positive or variable (Martelli *et al.*, 2003). A metabolic gender difference was found : male but not female hepatocytes gave UDS response (Joosten *et al.*, 2004), 32-P-post labelling was negative in rat liver *in vivo* (Feser *et al.*, 1996) SCE induction in human lymphocytes was negative in one study (Ahmad *et al.*, 2001), but positive in another study (Dhillon and Dhillon, 1996). Administration of norethisterone to mice caused SCE formation in bone marrow cells (Dhillon and Dhillon, 1996). Positive, negative and variable results have been obtained in tests for chromosomal aberrations or micronucleus induction with norethisterone (Dhillon and Dhillon, 1996; Stenchever *et al.*, 1969; Martelli *et al.*, 1998).

Norethynodrel (CAS No : 68-23-5)
(17 α)-17-hydroxyl-19-norpregn-5 (10) en-20yn-3-one)
Colour: White or almost white
Odour: Odourless

Solubility: Practically insoluble in water; soluble in alcohol, chloroform, dimethylsulphoxide.

Cancer studies: Norethynodrel (Fig. 12) was tested by oral administration in mice and rats and by subcutaneous implantation in mic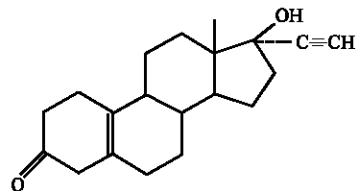e. It increased the incidence of pituitary tumours in mice of each sex and that of mammary tumours in castrated mates of one strain. It also increased the incidence of benign and malignant liver cell, pituitary and mammary (benign and malignant) tumours in male rats (Jordan *et al.*, 1993).

Genotoxic studies: Norethynodrel was negative for ames test (Lang and Reimann, 1993). For unscheduled DNA synthesis it was positive in male rat hepatocytes *in vitro*, but negative in rat hepatocytes *in vitro* (Joosten *et al.*, 2004). Norethynoderel did not induced aneuploidy in human cells in culture or unscheduled DNA synthesis in rat hepatocytes *in vitro* it inhibited inter cellular communications in Chinese hamster V79cells (Joosten *et al.*, 2004). It induced chromosomal aberrations and sister chromatid exchanges in the presence of metabolic activation supplemented with NADP in cultured human lymphocytes (Siddique and Afzal, 2005d; Siddique *et al.*, 2007d, 2006d) and chromosomal aberrations and sister chromatid exchanges at 15.62 and 31.25 mg g^{-1} body weight in mice bone marrow cells with a LD 50 dose of 125 mg kg^{-1} (Siddique and Afzal, 2003).

Dimethisterone (CAS No : 79-64-1)
(6α,17β)-17-hydroxyl-6-methyl-17-(1-propynyl)-adrst-4en-3-one)
Colour: White
Odour: Odourless

Solubility: It is insoluble in water, freely soluble in dehydrated alcohol, very soluble in chloroform, acetone, dimethyl sulphoxide.

Cancer studies: Dimethisterone (Fig. 13) was tested for its carcinogenicity in various animal models. There is no increase in tumour incidence (Drill, 1980).

Genotoxic studies: It did not induced chromosomal aberration in cultured human peripheral blood lymphocytes (Stenchever *et al.*, 1969).

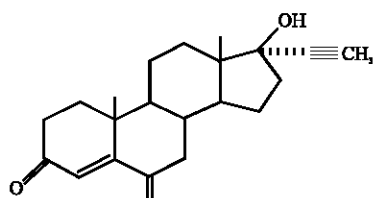**Progesterone (CAS No : 57-83-0)**
(Preg-4-ene-3, 20 dione)

Fig. 13: Dimethisterone

Colour: White or slightly yellowish white
Odour: Odourless

Solubility: It is practically insoluble in water, freely soluble in dehydrated alcohol, very soluble in chloroform, sparingly soluble in acetone, in ether, in fixed oils and dimethysulphoxide.

Postulated mode of action: Progesterone (Fig. 14) is used in oral contraceptives and in cure of premenstrual syndrome. It is 95-98% bound to plasma proteins and is metabolized as glucoronide conjugate by the liver (Schindler *et al.*, 2003).

Cancer studies: Progesterone was tested by subcutaneous and intra by intramuscular injection in mice, rabbits and dogs and by subcutaneous implantation in mice. It increased the incidences of ovarian, uterine and mammary tumours in mice. Neonatal treatment with progesterone enhanced the occurrence of pre-cancerous and cancerous lesions of the genital tract and increased mammary tumorigenesis in female mice (Lamb *et al.*, 2007). Genotoxic studies: Progesterone did not induce dominant lethal mutations in mice or chromosomal aberrations in rats treated *in vivo*. It did not induced chromosomal aberration or sister chromatid exchange in cultured human cells, nor chromosomal aberrations or DNA strand breaks in rodent cells. Studies on transformation of rodent cells *in vivo* were inconclusive; a clearly positive result was obtained for rat embryo cells, are weakly positive result for mouse cells and a negative result for Syrian Hamster embryo cells. Progesterone was not mutagenic to bacteria (Joosten *et al.*, 2004) The test results of the L5178-YTK mouse lymphoma assay were inconclusive (Myhr and Caspary, 1988), All data a UDS induction in male rat or human hepatocytes (Oshiro *et al.*, 1986; Martelli *et al.*, 2003) or ^{32}P-post labelling in the rat liver *in vivo* or human hepatocytes (Feser *et al.*, 1996; Werner *et al.*, 1997), indicate that progesterone does not cause DNA lesions variable responses with inter-animal differences in UDS were observed in male and female rat hepatocytes (Martelli *et al.*, 2003). Progesterone was not clastogenic in cultured human lymphocytes (Stenchever *et al.*, 1969).
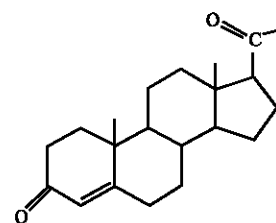

Fig. 14: Progesterone

Increased frequencies of micronuclei were found in hepatocytes of rats treated with a single oral dose (100 mg kg^{-1}) of progesterone (Martelli *et al.*, 1998). It does not induce an euploidy in DON cells *in vitro* (Wheeler *et al.*, 1986).

**MECHANISM FOR THE GENTOTOXICITY**

The carcinogenic effects of hormone replacement therapy used to relieve symptoms of menopause were evaluated by the International Agency for Research on Cancer (Joosten *et al.*, 2004). Most of the studies reviewed did not differentiate between the effects of estrogen only and estrogen-progestin combination therapies. An increased risk of endometrial cancer was associated with increasing duration of therapy (Kresowik *et al.*, 2008). Numerous case control and cohort studies have addressed the risk of various cancers associated with the use of oral contraceptives (Heinemann *et al.*, 1997). Most of the studies involved estrogen-progestins combinations. Studies in rats, mice, hamsters and guinea pigs have been conducted with estrogens alone or in combination with known carcinogens. Estrogen had a carcinogenic effect in all species and by all routes of administration. Most studies showed induction of benign and malignant neoplasias as well as preneoplastic lesions, in a variety of target organs, including the breast and female reproductive tract (Joosten *et al.*, 2004).

Steroidal estrogens can damage chromosomes and DNA in mammals (Banerjee *et al.*, 1994; Siddique and Afzal, 2004f; Siddique *et al.*, 2005b). The most frequently reported effects include DNA adduct formation, cytogenetic alterations (e.g chromosome and chromatid breaks, micronuclei, SCEs), aneuploidy and cell transformation (Djelic and Djelic, 2002). The genotoxic effects of estrogens/synthetic progestins/androgens have been demonstrated in various *in vitro* assays, using cultured animal cells or cell free systems. Fewer effects have been reported in whole animal studies or in studies with human cells and no human *in vivo* studies were identified (Siddique *et al.*, 2007e; Beg *et al.*, 2007; Joosten *et al.*, 2004; Djelic *et al.*, 2005).

Many different formulations of synthetic and naturally produced estrogens are prescribed for use as oral contraceptives or in postmenopausal hormone replacement therapy (Biri *et al.*, 2002). Exogenous estrogens are well absorbed from the gastro intestinal tract and the skin of human animals. Estrogens are metabolized in the gastro intestinal and other tissues. In both human and animals, estrogens undergo similar phase I and Phase II reactions. Aromatic hydroxylation reactions catalyzed by cytochrome P-450 enzymes are the primary phase 1 pathways. (Chen *et al.*, 1998). Sulfation, methylation and glutathione conjugation are the major phase 2 pathways. The major phase 1 metabolic pathway for endogenous estrogens is aromatic hydroxylation to catechol intermediates. Concerning the genotoxic effects of estrogens; 16α-Hydroxyestrone, 4-hdroxyestradiol and 4-hydroxyestrone have direct genotoxic effects and carcinogenicity. Liehr *et al.* (1986) described mechanistic similarities between human breast cancer and estrogen induced kidney cancer in hamsters and identified metabolism to the 4-hydroxylated catechols as the primary pathway leading to tumour development. The 4-hydroxylated catechols may undergo subsequent redox cycling between semiquinone and quinone forms. The quinones may undergo nonenzymatic isomerization to quinone metabolites. The quinone and quinone metabolites intermediates are highly reactive and may form covalent DNA adducts, thus these metabolites are candidates for ultimate estrogen carcinogens. Redox cycling between various forms of quinones generates superoxide radicals that are capable of direct and indirect damage to DNA. In breast cancer cells, 4-hydroxylation predominates over 2-hydroxyltion (Zhu and Conney, 1998).

Excessive production of reactive oxygen species has been reported in breast cancer tissue and free radical toxicity (DNA single strand breaks, lipid peroxidation and chromosomal abnormality) has been reported in hamsters treated with estradiol. Reactive oxygen species, including superoxide, hydrogen peroxide and hydroxyl radicals, may be produced through redox cycling between the O-quinones and their semi quinone radicals (Roy *et al.*, 1991). These reactive oxygen species can cause oxidative cleavage of the phosphate-sugar back bone and oxidation of the purine and pyrimidine residues of DNA. The incubation of 4-hydroxylated catechols with microsomes, NADPH and DNA resulted in 8-hydroxylation of guanine bases. 8-hydroxy-deoxyguanosine is a biomarker for oxidative damage and is considered an important factor in carcinogensis (Han and Liehr, 1994; Bolton *et al.*, 1998; Bolton, 2002). The possible mechanism and cause of the genotoxicity at different dosages of synthetic progestins such as cyproterone acetate, Chlormadinone acetate, medroxy progesterone acetate, have been studied by using different doses of superoxide dismutase and catalase in the presence of metabolic activation with and without NADP. Superoxide dismutases (SODs) are family of metal enzymes that convert O^{-}_2 to H_2O_2 according to the following reaction (Culotta, 2000).

$$O^{-}_2 + O^{-}_2 \xrightarrow[\text{SOD}]{2H} 2H_2O$$
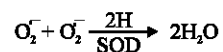
Catalase is a heme containing protein that brings about the decomposition of H_2O_2 into water and oxygen. Catalase is found to reduce sister chromatid exchanges levels. The mechanism of reaction is (Reid, 2003):

$$2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2$$

The treatment of SOD with ethinylestradiol in the presence of metabolic activation with NADP has been reported to increase the genotoxic damage (Siddique *et al.*, 2005b). Since ethinyl estradiol is genotoxic only in the presence of metabolic activation with NADP, the first step would involve the aromatic hydroxylation catalysed by cytochrome P450, as occurs in the case of estrone and the 17β-estradiol forming catechol metabolites (Yager and Liehr, 1996; Bolton *et al.*, 1998). Cytochrome P450, in liver S9 fractions plays an important role in activating promutagens to proximated/or ultimate mutagens. Rat and human liver P450 is involved in the activation of some chemical carcinogens having different isoforms (Maron and Ames, 1983; Guengerich and Shimada, 1991). In the tissues of liver, 2-hydroxylation predominates over 4-hydroxylation by approximately 9:1, however, in extra hepatic tissues the ratio drops to 1:1 (Zhu and Conney, 1998). Only 4-hydroxyestrone-3, 4-dihydroxy 1,3,5 (10)-oestraien-17one (4-OHE) was found to be carcinogenic in the male Syrian golden hamster kidney tumour model, whereas 2-OHE was found to be without any activity (Yager and Liehr, 1996; Guengerich and Shimada, 1991). Once formed, the endogenous catechol estrogens can be oxidized by virtually any oxidative enzymes in the absence or presence of metal ion and can give rise to o-quinones (Yager and Liehr, 1996; Bolton *et al.*, 1998). The study on ethinylestradiol confirms the presence of Reactive Oxygen Species (ROS) and the source of reactive oxygen species has been suggested to be the result of redox cycling between o-quinones and their semiquinone radicals, generating superoxide, hydrogen peroxide and ultimately reactive hydroxyl radicals, that cause oxidative cleavage of the
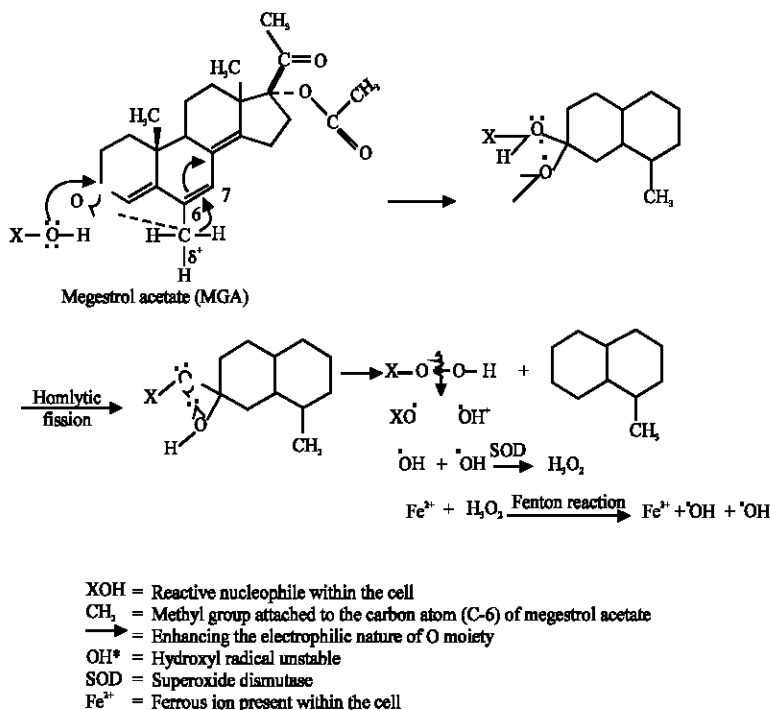
Fig. 15: Possible mechanism of generating free radicals by megestrol acetate (MGA) (Siddique *et al.*, 2005a; Hum Exp
Toxicol)

phosphate sugar backbone as well as oxidation of the purine/pyrimidine residues of DNA (Han and Liehr, 1994).

The study on cyproterone acetate, chlormadinone acetate and megestrol acetate reveal that the compounds increases chromosomal aberrations and frequencies of sister chromatid exchange, significantly in cultured human lymphocytes (Siddique and Afzal, 2004b, 2005a, b). The treatment with superoxide dismutase, increased the frequencies of chromosomal aberrations and sister chromatid exchanges generated by both cyproterone acetate and chlormadinone acetate. The possible mechanism of free radical generation for cyproterone acetate, megestrol acetate and chlormadinone acetate is shown in Fig. 15-17.

XOOH can also give rise XOO$^{\bullet}$ (alkoxyl) and $^{\bullet}$OOH (Peroxyl) radicals. The presence of alkoxyl (XOO$^{\bullet}$) radical appears to be a very remote possibility in view of the highly polar nature of the living system, because X would have to be essentially an alkyl group (Islam *et al.*, 1991). Hydroxyl radical (OH) is converted into hydrogen peroxide by superoxide dismutase (Culotta, 2000), as a result, the increase in chromosomal aberrations and sister chromatid exchanges was observed when the cyproterone acetate and chlormadinone acetate were

treated with superoxide dismutase. The catalase treatment with cyproterone acetate and chlormadinone acetate have been reported reduce the genotoxic damage (Siddique and Afzal, 2004b, 2005a, b).

Megestrol acetate has been reported to be genotoxic in mice bone marrow cells (Siddique *et al.*, 2005a). The treatment of megestrol acetate with ascorbic acid reduced the genotoxic damage of megestrol acetate. Ascorbic acid posses a substantial nucleophilic character and it has been suggested that ascorbate might protect against electrophilic attack a cellular DNA by intercepting reactive agents (Edgar, 1974). Reactive oxygen species generated by MGA via nucleophilic reaction have been suggested responsible for the genotoxic damage (Siddique *et al.*, 2005a). Norgestrel shows genotoxic effects only in the presence of metabolic activation in the presence of NADP (Siddique *et al.*, 2006c). The metabolic activation of norgestrel and possible conversion of it to reactive species may be responsible for its genotoxicity. Metabolic activation of estrogens such as estradiol-17β and ethinyl estradiol results in the production of reactive oxygen species via redox cycling between quinones and semi-quinone radicals (Fig. 18) (Siddique *et al.*, 2005b).
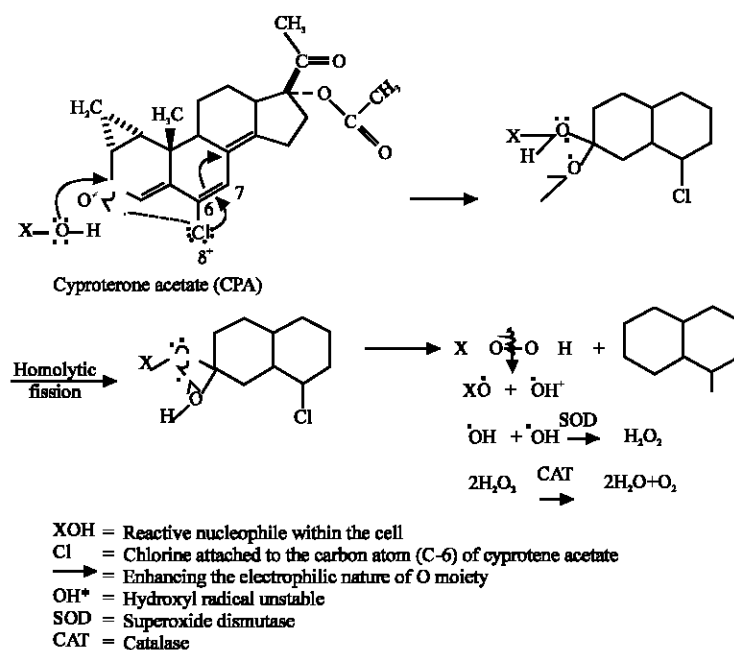
Fig. 16: Possible mechanism of generating free radicals by cyproterone acetate (Siddique and Afzal, 2005b; Toxicol *in vitro*)
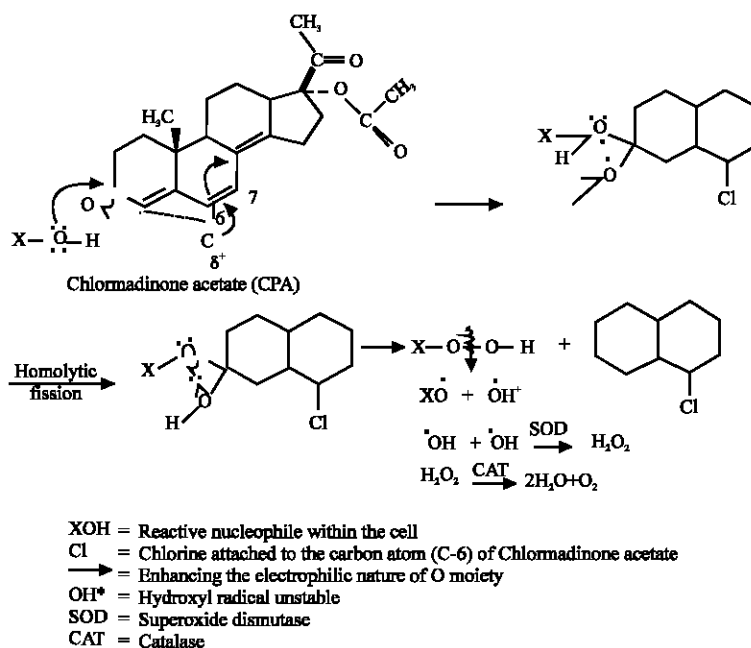

Fig. 17: Possible mechanism of generating reactive oxygen species by chlormadinone acetate (Siddique and Afzal, 2004b) Indian J Exp Biol

Medroxyprogesterone acetate (Fig. 19) showed genotoxic effects in the presence of metabolic activation supplemented with NADP (Siddique *et al.*, 2006a).The genotoxic potential of various synthetic progesting has been tested in different experimental models using different genotoxic end points (Joosten *et al.*, 2004). Very little attention has been paid to the structural relationship and the potentiality to induce the genotoxic damage by
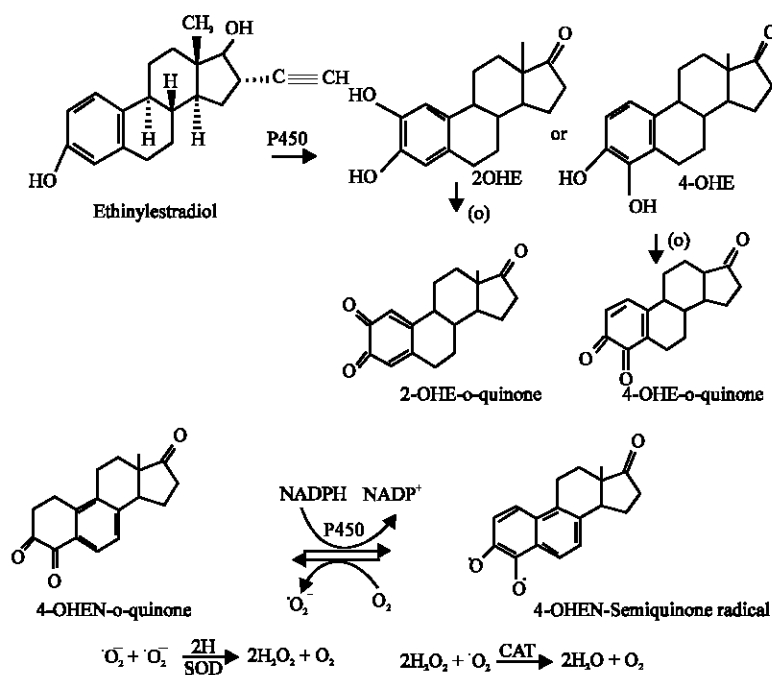
Fig. 18: Possible metabolism ethinylestradiol to catechols and quinones and generation of reactive oxygen species by redox cycling of 4-hydroxy equilenin (Siddique *et al.*, 2005b, Chem Biol Interact)
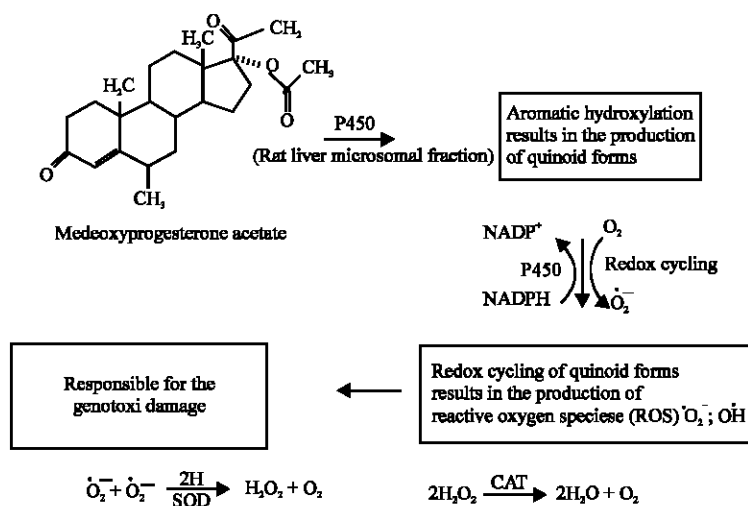

Fig. 19: Possible metabolism and generation of reactive oxygen species by medroxyprogestrone acetate (Siddique *et al.*, 2006a Life Sci)

synthetic progestins. Structural relationship and ability to induce genotoxic damage has been worked out, using DNA repair assay and micronucleus formation in the liver of female rats (Brambilla and Martelli, 1997). For DNA repair following trend was found, viz. cyproterone acetate>chlormadinone acetate>megestrol acetate. It was reported that cyproterone acetate with (a) a1, 2α-methylene group, (b) a keto group at carbon-3, (c) two double bonds, $C_4 = C_5$ and $C_6 = C_7$ and (d) Cl at carbon-6, is the most genotoxic molecule. The lower genotoxic potencies of chlormadinone acetate and megestrol acetate might well be due to the absence of 1, 2α-methylene group and for megestrol acetate, perhaps due to the presence of methyl group (-CH₃) at carbon-6 instead of Cl (Brambilla and Martelli, 1997). However, when the adduct formation in cyproterone acetate, medroxyprogesterone

acetate, megestrol acetate and chlormadinone acetate are considered, the cyproterone acetate, megestrol acetate and chlormadinone acetate are found to form adducts in human liver slices *in vitro*. Medroxyprogesterone acetate forms these at low level (Feser *et al.*, 1998).

Studies performed on megestrol acetate (Siddique *et al.*, 2005a), cyproterone acetate (Siddique and Afzal, 2005b), chlormadinone acetate (Siddique and Afzal, 2004b), ethynodiol diacetate (Siddique and Afzal, 2004c), norethynodrel (Siddique and Afzal, 2005d), norgestrel (Siddique *et al.*, 2006c) and lynestrenol (Siddique and Afzal, 2004e), show that the progestins, in which double bond between carbon-6 and carbon-7 is present, like megestrol acetate, cyproterone acetate and chlormadinone acetate, can undergo nucleophilic reaction and generate free radicals in the system and therefore, show the genotoxic effects and the progestins, in which double bond between carbon-6 and carbon-7 is absent, may require additional metabolic activation to show the genotoxic effects in the test system. Looking at the chemical structure of estradiol and ethinylestradiol, the double bond is found to be absent between carbon-6 and carbon-7. It is well known that estradiol undergoes

aromatic hydroxylation in liver by cytochrome P450s and results in the formation of catechol metabolites (Maclusky *et al.*, 1981; Fishman, 1983; Liehr *et al.*, 1986; Li and Li, 1987; Bolton, 2002). The redox cycling between O-quinones and their semi-quinone radicals results in the generation of superoxide, hydrogen peroxide and ultimately reactive hydroxyl radicals which cause oxidative cleavage of the phosphate-sugar backbone as well as oxidation of the purine/pyrimidine residues of DNA (Han and Liehr, 1994). Ethinylestradiol generates reactive oxygen species in the presence of metabolic activation with NADP (Siddique *et al.*, 2005b). The study performed on norgestrel, ethynodioldiacetate, lynestrenol and norethynodrel shows that they exhibit genotoxic effects only in the presence of metabolic activation with NADP. The similarity in the structure of the above synthetic progestins and the estrogens lies mainly in the absence of double bond between carbon-6 and carbon-7 (Fig. 20).

Moreover, if the adduct formation on the basis of presence and absence of double bond between carbon-6 and carbon-7 is considered, a definite correlation is established. A study, carried out by Feser *et al.* (1998) on
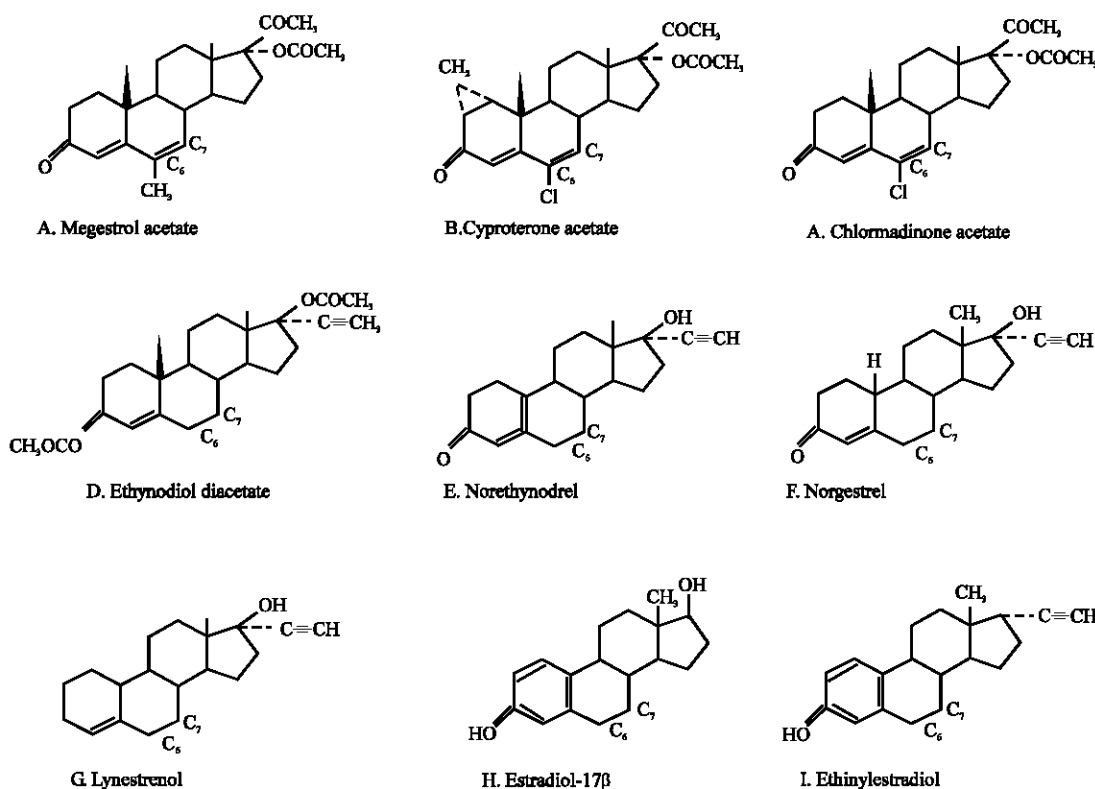

Fig. 20: Structure of synthetic progestins. Presence of double bond between carbon-6 and carbon-7 (A to C. Absence of double bond between carbon-6 and carbon-7 (D-I). (Siddique *et al.*, 2007c; Biomed Res)
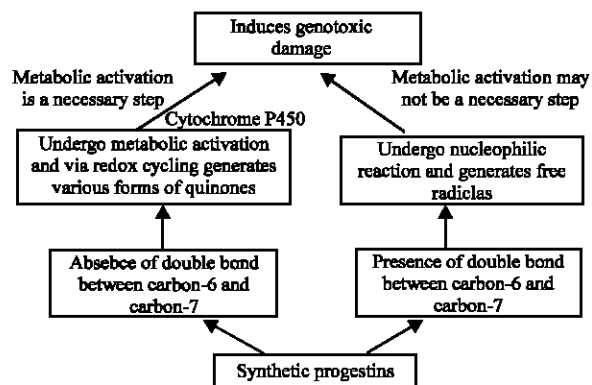
Fig. 21: A model for the genotoxicity of synthetic progestins

some selected sex-steroids, shows that chlormadinone acetate ($C_6 = C_7$) and megestrol acetate ($C_6 = C_7$) from DNA adducts, while norgestrel (C_6-C_7), estradiol (C_6-C_7) and ethinylestradiol (C_6-C_7) and ethinylestradiol (C_6-C_7) do not form DNA adducts in human liver slices. it is thus concluded that the double bond between carbon-6 and carbon-7 plays an important role in determining the mode of the genotoxicity of synthetic progestins. On the basis of above information the model for the genotoxicity of steroids (synthetic progestins/estrogens) is suggested as shown in the Fig. 21. Estrogens have been reported to induce genotoxicity in various *in vitro* and *in vivo* models. Synthetic progestins are at second position. Estrogens caused genotoxicity by metabolic activation i.e., forming catechol estrogens. The balance of toxification and detoxification determines the responsiviness by an individual and it is species and tissue dependent. The natural estrogen can cause DNA damage in the following ways (Joosten *et al.*, 2004).

- Redox cycling of catechol estrogen gives rise to reactive oxygen species (O_2^{-}) that can cause single strand break
- Hydroxyl radical can oxidized DNA base (Guanine)
- Lipid peroxidation may also take place and results in the formation of malonaldehyde DNA adducts
- Estrogen-DNA adducts may be formed after metabolic activation

For the synthetic progestin present study shows that the presence of double bond between carbon-6 and carbon-7 might be important for the genotoxicity. The presence of double bond is and the adduct formation is also correlated with the study performed on few selected synthetic progestins by Fesar *et al.* (1998). The

contradictory results for the studied synthetic progestins may be due to the low robustness of the various test models (Clastogenicity). *In vitro* conditions may generally favour phase-I activation reactions while not allowing phase-II de-activation reactions. The metabolic activation is a necessary step for the genotoxicity of natural estrogens as well as for the synthetic progestins. Some synthetic progestins are also genotoxic in the absence of metabolic activation (Joosten *et al.*, 2004). The dose is an important factor in determining the genotoxicity testing. Dose response relationship must be studied to reach any conclusion. Generally high doses of estrogens and synthetic progestins have been studied in various *in vitro* experimental models i.e., in µM and µg. The therapeutic plasma concentrations are often in the nanogram or pictogram mL^{-1} range. The high doses studied are significant because this dose range may reach in some clinical abnormal conditions or due to lack of metabolizing enzymes or other factors (Martelli *et al.*, 2003). As it is evident from the epidemiological studies that prolonged users of steroids, are at risk of having various types of cancers. The dose factor and the clastogeny as its own importance. An increase in the frequency of chromosomal aberrations in peripheral blood lymhocytes is associated with an increase in overall risk of cancer (Hagmar *et al.*, 1994, 1998).

CONCLUSION

The steroids are genotoxic only at high doses. The therapeutic doses are safe. The care should be taken with regard to their concentration as they may be carcinogenic in the long term use in humans.

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