CHAPTER IV

DISCUSSION

PART I

A total of 122 cases were investigated for various causes of male infertility. Endocrine, biochemical as well as seminal factors were investigated to probe into the possible causative factors that contributed to poor fertility in the patients screened. The patients investigated in the present study were classified into 4 groups as discussed earlier.

SECTION IA

Endocrine evaluation of the cases referred for various causes of infertility in the males and further correlation of these findings with clinical and cytogenetic studies helped to throw more light on the probable cause of infertility in the patients investigated in Gujarat.

GROUP I

The males investigated in this group were considered as controls. They were normal healthy males in the age group of 22 to 36 years with 2-5 years of married life and of proven fertility. Endocrine investigations revealed normal
ranges of all hormones assayed, viz. FSH, LH, T, PRL. Hence this group served as the control group and the endocrine investigations of all the other groups were compared to this group.

GROUP II

The males investigated in this group were diagnosed as azoospermic or primary sterility cases. Azoospermia was confirmed by semen analysis as discussed in Section II.

Among the 24 cases of azoospermia, 62.4% showed elevated serum gonadotropins with sub-normal testosterone levels. This data is in agreement with observations of others (Christiansen, 1975; Hopkinson et al., 1977; Pierrepoint et al., 1962). Highland (1989) and Pandya (1994) have also observed increased levels of gonadotropins in such cases. These results indicate primary testicular failure, with reduced germ cell proliferation and Leydig cell function. High FSH and LH levels distinguish primary testicular failure from hypothalamic – pituitary disorders (Swerdloff and deKrester, 1983) and reflect on testicular dysfunction, which leads to an alteration in the normal feedback inter-relationship between the hypothalamo-hypophysial gonadal axis (Bain et al., 1979). Consequently, testicular response to pituitary gonadotropin stimulation is impaired, as is evident in these individuals with absence or reduced spermatogenesis and low T levels. Klinefelter syndrome and its variants were detected by karyotyping. Eight out of the twenty four azoospermic males were confirmed to be Klinefelter syndrome cases with an extra X chromosome in their karyotype (47,XXY). The effect of varying numbers of X
chromosome on the development of sexual characteristics are of significance in the males. The presence of additional X-chromosome appears to interfere with normal gonadal development so that testicular function is impaired. As a result of this, the gonad is unable to respond to gonadotropin stimulation and endocrine function being greatly reduced, results in poor phenotypic development and spermatogenic arrest. Hence in patients with 47,XXY constitution, the tests are small and do not produce spermatozoa and therefore they are azoospermic. Klinefelter syndrome is commonly associated with a relative increase in the number of Leydig cells, as well as the characteristic degeneration observed in the seminiferous tubules (Balze et al., 1952; Howard et al., 1950 and Klinefelter et al., 1942). The relatively increased Leydig cells were considered to be immature and nonsecretory forms (Howard et al., 1950; Sinffen, 1952). This would appear to be supported by the finding of elevated gonadotropins and a tendency towards subnormal levels of T in these patients (Humphery et al., 1976; Paulsen et al., 1968 and Smals et al., 1974). The endocrine profile of the 8 Klinefelter syndrome cases investigated is in concordance with the above finding. Other workers (Paulsen et al., 1968; Wong et al., 1978; Ismail, 1981) have also shown similar data of endocrine patterns in Klinefelter males. Smals et al. (1981) have postulated that the elevated gonadotropin levels, resulting from desensitization of the Leydig cells through a mechanism of down regulation, is responsible for low secretion of testosterone in Klinefelter syndrome.

In 20.8% cases of the azoospermic patients, normal levels of testosterone, LH and FSH were observed. These patients probably have either retrograde
ejaculation or an obstruction of the ejaculatory system (Swerdloff and de Kreutzer, 1983).

In 4.16% cases of the azoospermic patients, endocrine evaluation revealed normal testosterone and LH with elevated FSH levels. This could be due to primary spermatogenic failure without associated Leydig cell damage. The elevated FSH is probably due to deficiency of inhibin in these patients (Anderson, 1974). This data is in agreement with Abyholm (1983) who reported abnormal elevated FSH levels indicating severe functional disorders in the germinal epithelium. Persistantly elevated FSH levels with normal LH and T values have also been demonstrated by other workers (Rosen and Weintraub, 1971; Franchimont et al., 1972 and Rodriguez-Rigau et al., 1978).

Elevated LH levels assayed in 8.3% cases suggested Leydig cell failure. In 4.16% cases of azoospermic patients had significant elevation in gonadotropins but T levels were normal which promoted a normal male phenotype and development of other secondary sex characters. In most infertile men, whether they are mildly oligozoospermic, severely oligozoospermic or azoospermic, circulating testosterone levels tend to be normal. Earlier report by Troen et al. (1970) showed that only 2 out of 11 men had mean testosterone levels below the normal range. Aiman et al. (1980) described three men with severe oligozoospermia or azoospermia who had reduced androgen receptor binding in cultured skin fibroblasts. These men had elevated testosterone production rates and gonadotropin levels. They had partial androgen resistance which appeared to play a role in the pathogenesis of their infertility.
Among the 16 primary sterility cases referred, 62.5% cases revealed a normal hormonal profile. This is in accordance with earlier work by Highland (1989) who also reported normal hormonal levels in 82% of males with primary sterility. In several cases, the condition may be idiopathic as elucidated in Section II, the infertility was found to be associated with significant alteration in semen characteristics.

In 12.5% cases of the patients referred as primary sterility revealed elevated FSH levels with normal T and LH levels which is suggestive of isolated spermatogenic compartment failure. Another 12.5% of the primary sterility cases showed low T levels with elevated gonadotropin which indicates primary testicular failure as discussed earlier.

In the remaining 12.5% cases referred for primary sterility, significantly elevated LH was seen with reduced T levels which suggests Leydig cell failure and impaired synthesis of the androgen. The testosterone levels however was sufficient to promote normal male phenotype and development of secondary sex characters. It is known that testosterone exerts significant control over libido, behaviour, accessory sex organs, epididymal function and spermatogenic processes (Ewing et al., 1980; Mawhinney, 1983). Hence decreased spermatogenesis, significant alteration in semen characteristics and infertility observed in these cases is the outcome of decreased androgen action.
GROUP III

The males categorised in this group comprised of those diagnosed as hypogonadotropic hypogonadism, gynaecomastia, oligozoospermia and varicocele. Among the 11 cases of hypogonadism, 36.36% cases showed normal levels of FSH, LH, T and PRL. However, significant reduction in sperm density (as described in Section II) was seen probably due to alteration of the germinal epithelium resulting in severe hypospermatogenesis.

In another 36.36% cases, a significant reduction in serum FSH and T levels were observed. Such subjects presented with sexual infantalism or incomplete sexual development (Spitz et al., 1974). Here, the germinal cell maturation of the testis may proceed normally, while testosterone secretion is negligible. These patients have been referred to as "fertile eunuchs" since spermatozoa may be present on testicular biopsy or in the ejaculate (Santen et al., 1971). A well documented case of isolated FSH deficiency was described by Mozaffarian et al. (1983).

In one case, non-detectable FSH with significantly elevated PRL was obtained. Prolactin acts upon the hypothalamus to increase dopamine turnover and thereby inhibit GnRH release.

Nine patients of Group III presented with moderate to marked gynaecomastia. Nuttal (1979) reported variable incidence of bilateral gynaecomastia, which ranged from 36% to 98%. Lee (1975) and Stanford (1983) have described the development of gynaecomastia as a common occurrence among boys who are virilizing rapidly, to be associated with a transient imbalance in the
T/E₂ ratio. Pathological gynaecomastia indicates an excess of estrogen over androgen activity, as was evident in the males of this study where significantly elevated E₂ levels (>70 pg/ml) were found in association with low T values (<2mg/ml). This resulted in a significant decline in the T/E₂ ratio, suggesting that estrogen action was probably responsible for gynaecomastia in these cases. Wilson et al. (1980) have classified gynaecomastia as due to reduced production of testosterone or due to increased production of E₂ through enhanced secretion or peripheral conversion from weak androgens. This observation supports our present data. Highland (1989) and Pandya (1994) also reported similar altered patterns of hormones in cases of gynaecomastia. The FSH and LH levels were on the lower border line except one case which showed significantly elevated FSH. Prolactin (PRL) levels were comparable to normal.

Seven of the Group III males were referred as oligozoospermic patients. Endocrine evaluation revealed a normal hormonal profile in these patients. In fact this hormonal pattern (normal FSH, LH and T) is seen in majority of the oligozoospermic patients who are categorised as having idiopathic oligozoospermia. The possibility of defective gonadotropin secretion, partial genital tract obstruction and androgen resistance as causes of oligospermia were less likely to be common etiological factors in infertile men. Other aspects eg. disorders of androgen biosynthesis have been associated with hypospermatogenesis (Steinberger et al., 1974; Rodriguez-Rigau, 1978), but these are not common (Nieschlag et al., 1979). Disorders of sperm maturation and extrinsic abnormalities of sperm motility could be a causative factor for poor fertility (Section II).
Varicocoele is the most common etiological factor demonstrable in subfertile men (WHO, 1984). The incidence of varicocoele in men presenting with infertility is quite significant. Cockett et al. (1979) have shown 41%, Aafyeser Van der Vijver (1985) 21%, while Marks et al. (1986) 34% men with infertility having varicocoele. In the present study two cases in Group III were diagnosed as cases having varicocoele. They showed normal levels of FSH, LH, T and PRL. Swerdloff and Walsh (1975) reported normal levels of FSH, LH, T and E₂ in varicocoele patients which is in agreement with our data. Others, however, have found a significantly higher serum testosterone in the varicocoele group (Comhaire and Vermeulen, 1975).

GROUP IV

Eighteen males were categorised in the miscellaneous group with various sexual disorders. They were further grouped into the adult group and pre-adolescent group according to their age. In the adult group there were 11 cases while 7 cases were reported in the preadolescent group. Of the 11 cases in the Group IV-A, 2 patients were referred for delayed puberty. While normal pubertal development occurred by the age of 14 in 97% boys, 3% do not show pubertal changes (Brook, 1982). The 2 patients (21 and 17 years old) in the present investigation, had short stature and low body weight and underdeveloped secondary sex characters. These observations could be correlated with the normal serum FSH and LH but significantly low T levels, suggestive of total androgen deficiency.

Puberty is initiated by an activation of previously quiescent hormone action.
The sensitivity of the negative feedback interaction between the gonads and the hypothalamo-pituitary axis change during puberty (Objeda et al., 1980; Kulin and Santen, 1982; Kulin and Maruca, 1983). Delayed onset of puberty probably results from delayed activation of the hypothalamo-hypophyseal axis. Brook (1982) on the other hand, have shown that late pubertal development may result from testicular damage which causes insufficient production of T and therefore absence of masculinization.

Two cases of homosexuality was referred in a 21 and 23 year old patient. Their endocrine evaluation revealed normal hormonal profile except for reduced T levels in one case. Homosexuality is more of a psychological problem related to a person’s sense of sexual identity and sexual preference. Mai et al. (1972) uncovered issues of sexual identity conflict as a significant psychological issue in their comparison of infertile and fertile couples.

One case of intersex (Case No. 1470) was reported wherein significantly elevated FSH and LH was obtained with low T and PRL levels. The elevated FSH and LH indicates primary gonadal failure, which corroborates with the clinical findings of small testicular size. The significantly low T levels can also be correlated with impaired differentiation of the external genitalia and the ambiguity in sex expression in this individual.

Two cases of pseudohermaphroditism were reported in a 16 and 22 year old individual. One case showed normal levels of FSH and LH with low borderline T while the other showed significantly low gonadotropins and T levels. The low T levels obtained in the present study may be sufficient to bring about development...
of male phenotype but insufficient to stimulate complete virilization of the external genitalia.

Ambiguous genitalia was reported in one case of a 33 year old man who had showed normal levels of hormones. Hypospadias was observed in 2 cases. Hypospadias without other abnormalities of sexual differentiation is the most common form of male pseudohermaphroditism and occurs approximately in 5:1000 male births (Belman and King, 1976). The causes of hypospadias and ambiguous genitalia have been attributed to deficient virilisation of the external genitalia, secondary inadequate androgen production, improper timing of the stimulus, androgen resistance or inadequate conversion of testosterone to its more potent metabolite, 5α dihydrotestosterone (DHT). In the 2 cases investigated, one case showed elevated hormonal profile while the other showed normal FSH, LH and T. One of the possible causes related to this disorder is thought to be due to early cessation of testosterone production by the foetal testis, with relative deficiency of the androgen receptor which consequently results in hypospadias (Wilson et al., 1974).

Seven patients of Group IV-B were categorised as pre-adolescent males in the miscellaneous group. They were referred for precocious puberty, delayed development, micropenis and abnormal sexual development. Low basal hormonal levels were detected in almost all the cases studied.
SECTION I B

HAEMATOLOGICAL STUDIES

The haemoglobin levels, blood glucose and serum cholesterol levels were assayed in all the patients referred for various causes of infertility and it was found that there was no significant alteration in any of these parameters investigated in any of the groups as compared to control. Therefore more than endocrine and metabolic factors, semen characteristics play an important role in contributing to poor fertility as is discussed in details in Section II.

SECTION II

SEMEN ANALYSIS

Semen analysis was carried out in all the groups of infertile males and compared to the control group. Several authors (Belsey et al., 1980; Aitken et al., 1982; Moghissi and Wallach, 1983) have stressed the importance of quantitative and qualitative evaluation of human semen. Currently dynamic tests aimed to determine the functional status of the different components of the spermatozoon have been invaluable to determine the fertilizing potential of a semen sample as well as to identify the causes of poor semen quality (WHO, 1991). Computer assisted semen analysis was also carried out concurrently with routine semen analysis because it gave details of sperm forward-progressive motility which again play a very important role in the fertilizing potential of a semen sample.

The significant reduction observed in the volume of semen of azoospermic and oligozoospermic males suggests reduced accessory gland function, since it is
known that the accessory glands, particularly the seminal vesicle, contributes the major volume of seminal plasma (Lundquist, 1949). The secretory activity of the accessory glands moreover, is under androgen control and the reduction in semen volume can therefore be attributed to low testosterone levels assayed in these cases. Zaneveld and Jayendran (1988) have considered low volumes to be due to an obstruction of the ejaculatory ducts or impairment of accessory gland function. Gopalakrishnan et al. (1992) have also reported reduction in semen volume in infertile cases.

The pH of the semen from the infertile groups did not show any alteration when compared to control group. Makler et al. (1981) have stressed the importance of pH as a factor for sperm motility and viability. The present study however did not show any significant alteration in the pH of semen from infertile and control group. The pH of the cervical mucus has been shown to be an important parameter of mucus quality with significant influence on spermatozoal viability in cervical mucus, which correlates with peripheral hormonal status (Waltraud et al., 1993).

Viscosity of the semen also were not markedly disturbed according to the generally accepted reference values (Gow, 1986). Unaltered viscosity was also reported by Omer (1993) in subfertile males which is in agreement with our data.

The limitation of conventional semen analysis as a means of assessing the fertilizing ability of the human spermatozoa has been repeatedly documented (Jeulin et al., 1988). Recently, investigations have been centered around determination of changes in the sperm nuclear chromatin content, which may affect
sperm morphology and fertilizing ability. Tejada et al. (1984) and Highland et al. (1991) have described a fluorescent staining technique for evaluating the nature of the sperm nuclear DNA. Tejada et al. (1984) had shown that a higher count of green fluorescing sperms was related to an increased number of ‘fertile’ cells in the sample. The results of the present study however did not reveal any significant alteration in the percent green fluorescing sperms in the control and infertile groups suggesting that the sperm nuclear integrity was not altered. This finding is contradictory to earlier observations by Ibrahim and Pederson (1988) and Highland (1989) who demonstrated a significant increase in the percentage of red fluorescing spermatozoa in the infertile semen as compared to the control.

The aqueous silver nitrate staining technique described by Bongso (1983) was specifically modified in our laboratory (Chinoy et al., 1992) using instead, an alcoholic acidic silver nitrate stain. The staining is based on the high affinity of silver nitrate for disulphide linkages in the sperm membrane. The differential staining with less intensity at the acrosome, is therefore related to the lability of the acrosomal membrane having less disulphide linkages. The staining demonstrates acrosomal integrity. Morales et al. (1988) have proved that normally shaped spermatozoa have normal movement characteristics with higher linear velocity than abnormally shaped germ cells. However, in the present study, an insignificant alteration was obtained in the percent abnormal sperms in the infertile groups as compared to control. A slight increase in the percent of morphologically abnormal sperms in the primary sterility cases with low sperm count was observed which however was not very significant. The morphologically abnormal sperms

111
included macrocephalic, microcephalic, amorphous, pyriform head, tail anomalies such as bent, coiled or split tails, presence of cytoplasmic droplets, sperm heads with dimunitive or lack of acrosome, bent neck or deflagellated spermatozoa. Kruger et al. (1988) have elucidated the importance of sperm morphologic features in the fertilizing and penetrating ability of spermatozoa.

The sperm mitochondrial activity index was determined to see if lack of mitochondrial enzymes which impairs sperm motility could be a factor responsible for impaired fertility in the infertile cases referred. In the present study, no alteration was found in the sperm mitochondrial index of the infertile and control groups. Therefore, the sperm metabolic and energy generating processes were not altered.

Semen analysis has been specifically focussed on determination of sperm concentration, viability, motility, morphology and biochemistry (Hudson et al., 1987). Sperm motility and sperm motion characteristics have been regarded as some of the most important parameters in evaluating fertility potential of a semen specimen. Recently, computer assisted semen analysis (CASA) has been emerging as a powerful tool for quantitative description of sperm motility, velocity, forward progressive motility and head movement (Gill et al., 1988). Currently such systems are also being increasingly employed in our country to monitor semen profiles. However, discrepancies have been demonstrated in the evaluation of sperm concentrations using such computer automated systems (Katz and Davis, 1987; Knuth and Nieschlag, 1988). In the present study, therefore, both conventional and computerized semen analysis have been carried out to to compare
the results of both conventional and automated analysis in the evaluation of fertility.

Among the 16 primary sterility cases investigated, majority of the patients showed normal serum hormone profile. Therefore, assessment of the semen was undertaken by both manual and computer-aided methods to identify semen related causes for poor fertility.

Determination of sperm count using a haemocytoueter indicated that Group II-A individuals had counts that were sub-normal but however, higher than the oligozoospermic range. On the other hand, Group II-B patients had sperm counts comparable to that of normal.

Computer automated semen analysis also revealed a similar trend in sperm concentration, with a significant reduction in sperm count in Group II-A individuals but no significant alteration in Group II-B individuals as compared to control men.

It was observed however, that the values obtained for sperm count by the automated cell soft system were higher than that analysed by the manual method. This discrepancy could be attributed to the increased count given by the computerised programme. Katz and Davies (1987) and Knuth and Nieschlag (1988) have demonstrated that major discrepancies exist in the analysis by automated systems. These workers have observed that in many instances, the computerised systems fail to distinguish debris from normal spermatozoa, leading to an over-estimation of sperm concentration. Hence, computer aided semen analysis should be validated by parallel manual haemocytometric examination for determination of sperm counts.
Sperm motility evaluated by both methods was observed to be significantly lower than normal in both the groups studied. The computer-aided system however provided vital information regarding the sperm forward progressive motility. It was observed, that although the sperm density was normal in semen in individuals of Group B, the forward progressive motility of spermatozoa was significantly lower than normal. Poor forward progressive motility results in impaired movement of the sperm along the reproductive tract. Progressive motility was an independent attribute of the sperm and was not found to be correlated with sperm density. In these patients, the forward progressive motility of spermatozoa was impaired. These findings are in agreement with the observations of Vantman et al.(1988) who reported that automated computer-assisted technologies offer a major advance in evaluating sperm motion characteristics.

The live:dead ratio of spermatozoa was lower in semen of Group II-B individuals, despite reports of normal sperm count and near normal motility. Hence, poor sperm viability and impaired forward progressive motility contribute to lower semen quality standards in these individuals.

In the Group III individuals comprising of oligozoospermic men also a similar trend was obtained. The sperm concentration and motility did not show very significant alteration, however, a very significant alteration was obtained in the forward progressive motility. Correlated with this, a significant reduction was obtained in the sperm viability.

From the investigations carried out in the infertile cases referred, it is quite clear that in the azoospermic males, endocrine imbalance played a significant role.
However, in the oligozoospermic and primary sterility cases, it was observed that rather than endocrine or metabolic disorders, semen quality standards played a more significant role in determining the fertilizing potential. The computer assisted semen analysis in particular was important in giving details of sperm motion characteristics and in the present investigation revealed that poor forward progressive motion of the spermatozoa could be one of the causative factors leading to sub-fertility in otherwise normal samples.

The present data, therefore, helps in understanding the causes of infertility in males in Gujarat state and its vicinity and aids in diagnosis and provides therapeutic regimen in these cases as well as counselling to help overcome the complex life crisis of infertility.
PART II

The efficacy of benzene and alcoholic extracts of ripe Carica papaya seeds as a contraceptive agent was tested on male albino rats (Rattus norvegicus). The extracts were administered orally at a dose of 20 mg/Kg body weight/day/30 days.

Earlier work carried out in our laboratory (Chinoy and Sam George, 1983; Chinoy and Geetha Ranga, 1984; Chinoy et al., 1984/1985, 1994, 1995a) revealed that the aqueous extract of ripe papaya was potent in reducing sperm motility and fertility in male rats. The effects of aqueous extract of Carica papaya as an antifertility agent in male rats, mice and guinea pigs have also been studied together with its toxicological investigation. Lohiya et al. (1992) have reported the contraceptive effects of orally administered crude chloroform extract in male albino rats. Hence, the present study was undertaken to investigate the contraceptive efficacy of the benzene and alcoholic extracts of papaya seeds on male albino rats and the possible reversal of the effects, if any. Some specific toxicological parameters were also investigated.

The extract was administered by the oral route as specified by WHO MB-50 protocol. Earlier work from our laboratory (Chinoy and Sam George, 1983; Chinoy and Geetha Ranga, 1984; Chinoy et al., 1984/1985, 1994, 1995a) has revealed that intramuscular administration at a dose of 0.5 mg and 5.0 mg/Kg body weight/day/animal of the aqueous extract was effective as an antifertility agent in male rodents. However, a slightly higher dose was required to bring about the desired effects when oral administration was given. Various doses (5, 10, 15 and
20 mg/Kg body weight/day/animal) were used and it was found that 20 mg/Kg/body weight/day/animal was most effective in causing fertility impairment and therefore this dose was selected for the present investigation.

The effects of the extract on sperm motility, density, morphology and fertilizing ability were studied in particular. All the parameters were assessed according to WHO protocol MB-50, which specifically tests the antifertility effects of plant products in rodents.

As required by the WHO guideline, the extract was first tested for its estrogenticity, if any, according to WHO protocol MB-70/71. The ovariectomised, extract treated immature female rats did not reveal any estrogen dependent changes such as increase in body weight, uterine and adrenal weights, presence of vaginal opening and epithelial cells in the vaginal smear. The study revealed that benzene and alcoholic extracts of papaya seeds were non-estrogenic when tested in immature albino female rats which is in agreement with earlier data for aqueous extract (Chinoy et al., 1984/85, 1994). An estrogenic effect is an undesirable effect in the formulation of a male contraceptive agent, since estrogenic compounds are known to manifest antiandrogenic and antigonadotrophic effects in the males, besides causing certain unwanted side effects and therefore the fact that both the benzene and alcoholic extracts do not possess estrogenic effect is of advantage.

Estrogens are also known to inhibit testicular and accessory sex gland functions (Bartke et al., 1977; Johnson and Gomes, 1977; Chinoy and Rao, 1982; Chinoy et al., 1982; Rao and Chinoy, 1984; Chinoy et al., 1984). Besides, the antifertility effects of most of the plant products is ascribed to their estrogenicity.
The LD₅₀ dose was 15 g/Kg body weight (Chinoy et al., 1994) which is much higher than LD₅₀ dose of 5 g/Kg body weight recommended by the WHO as of non toxic nature for a plant product. This revealed that both the extracts were non-toxic. Moreover, some other toxicological parameters have been investigated and the results discussed in a later part of the discussion.

The parameters considered for assessing the antifertility effect of the benzene and alcoholic extracts were sperm motility, forward progressive motility, sperm count, viability and fertility rate. Moreover, certain biochemical parameters were also studied viz., the DNA, RNA and protein levels in testis, cholesterol, 3β HSD and 17β HSD levels in testis to elucidate the effects of the extract on the steroidogenic pathway. The sperm acrosomal enzymes viz. acrosin and hyaluronidase were investigated as they are important enzymes for sperm penetrability which is a prerequisite before fertilization. Besides some sperm functional tests like sperm mitochondrial activity index, acridine orange staining was also carried out to assess the nuclear integrity. Serum testosterone levels were assessed by RIA to determine the possible effect of the extracts on the testicular steroidogenesis. The levels of serum Na⁺, K⁺ and Ca²⁺ ions were also investigated using flame photometry. The reversibility of the induced effects by withdrawal of treatment for a period of one month after 30 days of administration of the extract was also investigated.

The body weights of all the treated animals (with both the extracts) were not affected following treatment suggesting that the extract does not promote body weight gain probably due to water and electrolyte retention. This is important
since Na⁺, K⁺ balance is necessary for maintaining the proper micro-environment particularly of the epididymis for sperm maturation (Wong et al., 1978; Chinoy, 1984). Correlating with this, the electrolyte balance was also maintained as evident from the unaltered levels of Na⁺, K⁺ and Ca²⁺ ions in serum of both benzene and alcoholic extract treated animals. Maintenance of electrolyte balance is important as changes in the level of sodium and potassium may cause loss of water from the cells and tissues leading to reduction in body weight. Alterations in serum calcium levels would affect the various reactions catalyzed by the enzymes and numerous calcium dependent functions of the body such as blood coagulation, muscle contraction, sperm motility, etc. would be affected. Sondarva (1989) has also shown unaltered serum Na⁺ and K⁺ levels following aqueous extract of papaya seed treatment in which agrees with the present data. Thus the data suggests that the osmotic and electrolyte balance was maintained.

The weights of the testis also remained unaltered following 30 days treatment by both the extracts. Earlier studies with aqueous extract (Chinoy and Sam, unpublished observations; Chinoy and D’souza, unpublished observations) have shown that the histoarchitecture of testis was not altered and that spermatogenesis was normal. In the present study, the sperm count was not affected after treatment with benzene extract, while treatment with alcoholic extract resulted in a decrease in sperm count. Earlier work has also reported unaltered sperm counts in rats and mice (Chinoy et al., 1994, 1995a) with aqueous extract of papaya seed which suggests that spermatogenesis was not altered in treated animals.
The cholesterol levels in the testis of rats remained unaffected following 30
days treatment with benzene extract of papaya seed. However, a slight decrease
was observed in the alcoholic extract treated group. The almost unaltered
cholesterol levels suggests that there is risk of neither hypo or hyper
cholesterolemia. Similar results have been obtained in mice (Chinoy et al., 1995a)
using aqueous extract of papaya seed. The activities of 3β and 17β hydroxysteroid
dehydrogenases which are the key enzymes in the steroidogenic pathway taking
place in the testis were not altered following both benzene and alcoholic extract
treatments. This data suggests that steroidogenesis is not hampered. From the
above observations, it is clear that the extract treatment did not interfere with
cholesterol synthesis and its subsequent turnover to the end products, viz. steroid
hormones. The normal serum testosterone levels in both the extract treated rats
corroborate with our above observation and with earlier data (Chinoy et al.,
1984/85, Chinoy et al., 1995a).

The serum FSH and LH levels were also within the normal range following
both the extract treatments (Chinoy and Joshi, unpublished observations) which is
again in accordance to earlier data (Chinoy et al., 1984/85). Therefore it is evident
that the pituitary gonadal axis is not altered suggesting that the extract has no
antigonadotrophic effect, which supports our data that the extract is non-estrogenic.
Based on these observations, it is clearly evident that both the extracts have no
effect on spermatogenesis, steroidogenesis nor pituitary gonadal axis. Thus the
extract manifested a clear post-testicular action.

The motility of cauda epididymal spermatozoa were reduced significantly
following both benzene and alcoholic extract treatments to rats. Assessment of sperm motion characteristics by computer assisted semen analyser (CASA, Cellsoft 2000) revealed a significant decline in the forward progressive motility of the sperms. Lack of forward progressive motility is one of the factors resulting in reduced fertility because only actively forward progressive motile spermatozoa are capable of penetrating and ascending the female reproductive tract. The type of movement of the sperm thus influences the fertilizing capacity. Sperms swimming in tight circle cannot readily pass through the uterotubal junction, and only straight swimmers succeed in fertilizing the egg (Blandau and Rumery, 1964). Correlating with the reduced motility and forward progressive motility, a slight decrease was also noted in the sperm mitochondrial activity index (SMAI) following benzene extract treatment. From the above observations, it is evident that both the extracts of papaya seed interfered with sperm motility. The extract possibly disrupts the -S-S linkage causing alterations in its membrane particularly those of the acrosome. The role of acrosome in fertilization is well established as it contains a number of enzymes such as acrosin, hyaluronidase, β-glucuronidase, acid and alkaline phosphatases which play a significant role in egg penetration by the spermatozoa. Acrosin and hyaluronidase are two principal acrosomal enzymes required for the acrosomal reaction before fertilization. A decrease in sperm acrosomal enzymes was reported to be one of the reasons for the lower fertilizing capacity of semen (Amelar and Dubin, 1979). Hence any damage to the acrosome may result in loss of these enzymes rendering the sperms non viable. Acrosin is a neutral protease instrumental in the penetration of the zona pellucida and cervical mucus by the
spermatozoa (Mc Rorie and Williams, 1974; Schumacher and Zaneveld, 1974). It occurs partly in the form of an inactive zymogen precursor, proacrosin (Polakoski et al., 1977). Assay of acrosin therefore, involves assay of zymogen precursors and activation of acrosin after removal of inhibitors, to quantitate total acrosin (Polakoski et al., 1977; Bhattacharya and Zaneveld, 1978). In the present study, proacrosin levels increased following benzene extract treatment. However, alcoholic extract treatment did not show any alterations. The level of acrosin-acrosin inhibitor complexes declined in both benzene and alcoholic extract treated animals. This suggests a block in autoactivation of proacrosin. Schill et al. (1988) has reported that activation of proacrosin levels might be due to alterations in glycosaminoglycans (GAG) concentration as GAGs are known to stimulate the conversion of proacrosin into its active forms (Parish et al., 1980). The decline in acrosomal enzyme could be correlated with the reduced fertility. Hyaluronidase disperses the cumulus oophorus and the spermatozoa uses their enzyme in penetration of the outer most layer of the ovum (Zaneveld et al., 1973). Hyaluronidase is also associated with the acrosome of the spermatozoa (Morton, 1975; Gould and Bernstein, 1975). A significant reduction in the hyaluronidase levels following 30 days treatment with both benzene and alcoholic extract could be related to the low penetrating ability of sperms and subsequently reduced fertility. Amelar and Dubin (1979) reported decrease in sperm acrosomal enzymes as one of the reasons for the lower fertilizing capacity of semen. Hence any damage to the acrosome may result in loss of enzyme rendering the sperms non-viable. A significant decrease in the acrosomal enzymes, hyaluronidase and
acrosin obtained in the present study is in confirmation with the above observations and therefore both the extracts rendered the sperms non-viable. This is further supported by the fact that there was a significant decline in the percentage of live sperms following both the extract treatment which in turn reduced the fertility.

The benzene and alcoholic extract treatments brought about a significant reduction in the fertility rate of normally cycling female rats mated with the treated males. The reduction in fertility is correlated with the significant decrease in percent cauda epididymal sperm motility, loss of viability and acrosomal damage.

It is known that RNA synthesis or activity of RNA polymerase in reproductive organs are androgen dependent (Williams Ashman et al., 1964). The increased levels of RNA in testis after treatment with both benzene and alcoholic extract may be due to primary and secondary diffuse atrophy of interstitial cells, germinal epithelium and crowding of cells with atypical nuclei. Increase in RNA synthesis or activation of RNA polymerase could possibly result in augmentation of the RNA concentration. This aspect needs further detailed study.

The significant increase in the DNA levels in testis following both the extract treatments could be due to rapidly proliferating cells or due to atrophide cells. The increase in DNA concentration could also be attributed to unscheduled DNA synthesis. In order to investigate the reversibility of the induced effects by the treatments, a group of animals were treated with extracts and thereafter the treatment was withdrawn and the reversibility was studied after a period of one month. The findings indicated that all the induced effects, i.e. reduction in sperm motility, the biochemical parameters assessed as well as the rate of fertility.
recovered significantly to almost the normal levels. Thus the extract possesses reversible antifertility effects without any apparent toxic side effects. Hence functional sterility could be induced in rats by benzene and alcoholic extract treatment which is in concordance with earlier data of Chinoy et al. (1984/85) in aqueous extract of papaya seed.

The present study clearly elucidates that the benzene and alcoholic extract of papaya seed is a potent, post-testicular male contraceptive agent which is non-estrogenic with complete reversibility of the induced effects, without causing any toxic side effects in the animals. The anti-implantation and anti-fertility action in female rats have also been studied in our laboratory (Chinoy et al., 1995b).

The present research work therefore makes a significant contribution in the search of a safe, effective and easily procurable oral male contraceptive agent with complete reversibility of the induced effects and offers a major lead in the field of male contraception.