Biochemical and Hormone Profiles in Cervical Cancer

By

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Introduction

I INTRODUCTION

Cancer is a disease of tragic dimensions. It is a growth of abnormal cells. Usually cell division is governed by a complex set of controls. When these controls fail, a normal cell becomes an abnormal cell in which the ordered cell growth, division and differentiation are lost. Instead, the cells begin to divide and multiply relentlessly, proliferating and massing into a tumor or cancer (Das and Mukhtar, 1985).

Carcinoma of the uterine cervix is the commonest malignancy seen among women (Singh et al., 1983). Adenocarcinoma of endometrium ranks second among the common forms of cancer of the reproductive organs (Winston and Woodard, 1982).

In developing countries, invasive cancer of the uterine cervix accounts for approximately five percent of all cancer deaths in women. Cervix cancer has been shown to occur more frequently in women of urban residence of young age at first intercourse and of low family income level (Stanley, 1981).

Early marriage, mixed sexual partners and poor penile hygiene also play a role (Novak and Woodruff, 1974). Cervical cancer has also been associated with women

who have borne many children although no definite relationship has been confirmed (Sebastian, 1983).

It has been known that human cancer is associated with abnormal production of a variety of circulating hormones, proteins and enzymes (McIntire, 1984). High levels of circulating immune complexes (CIC) have been reported in the sera of patients suffering from malignant diseases including carcinoma of uterine cervix (Sharma et al., 1985). The association between Herpes Simplex virus and cervical cancer was well known (Kumari et al., 1982). Sixty four and a half percent women with cervical carcinoma had Herpes Simplex virus -2 (HSV-2) antibodies against 34.8 and 36.7 percent women with cervical dysplasia and controls respectively (Luthra et al., 1983).

There were several immunological abnormalities in cervical cancer. There was a significant rise in serum IgG levels in carcinoma cervix patients ($P < 0.001$). The level ranged from 1000 to 2100 mg percent with a mean 1555.8 mg percent ± 263.18. Serum IgA levels varied from 140 mg percent to 420 mg percent with a mean of 275.5 mg ± 75.60. The value of IgM ranged from 100-280 mg percent with a mean of 179.58 ± 42.5 mg percent (Sharma et al., 1979).

The mean concentration of ascorbic acid in the plasma was significantly lower in the cases than in the controls (0.36 against 0.76 mg/dl) (Ramsay, 1986). Plasma folate and B-carotene were also found to be significantly lower in patients with cervical cancer when compared to controls (Orr et al., 1985). Haemoglobin levels in carcinoma cervix patients were also significantly lower than the control group and there was a graded reduction in the haemoglobin levels with the advancing stages of the disease due to excessive bleeding (Sharma et al.,1979).

The activities of aspartate amino transferase and alanine aminotransferase in erythrocytes with and without the addition of pyridoxal phosphate were estimated in healthy controls and in Indian women with cancer of uterine cervix. In patients with cervical cancer, 23-35 percent stimulation was observed indicating a deficiency of vitamin B6 where as in normal subjects it was negligible (less than 5 percent) (Ramaswamy and Natarajan, 1984).

It has been estimated that in women, 60 percent of all cancer is related to diet (Watson et al., 1986). Serum selenium levels in patients with cervical cancer increased ($P < 0.001$) from 1978-1982. The values were 0.71 ± 0.03; 0.87 ± 0.05 and 1.16 ± 0.03 during the years 1978-79; 1980-81 and 1982 respectively. This increase appeared to correlate with the estimated daily intake

of selenium during these years. The level decreased in 1983 (1.06 ± 0.07) when the amount of selenium rich grain was reduced (Sundstrom, 1986).

It had been shown that the levels of acid labile DNA were raised in the cervical smear of the cancer patients (Sinsook, 1986).

In double blind condition, 47 young women with mild or moderate dysplasia of the uterine cervix were given supplements by mouth 10 mg folic acid. Mean biopsy score of subjects given folate, were significantly better than in subjects given not folate supplement. Final against initial cytology scores were also significantly better in subjects given folate supplement (Butterworth et al., 1983).

High doses of vitamin C given everyday without stopping as an adjunct to conventional therapy improved health and survival time of cancer patients (Pauling and Moertel, 1986).

In the present study, twenty one women with cervix cancer were studied. These cases were evaluated for plasma ascorbic acid and serum cholesterol, total creatinine, total protein, albumin, globulin, zinc, copper, iron, manganese, prolactin and estradiol. These values were then compared with those of nine normal women who served as positive controls.

The aim of this study was to find out the biochemical abnormalities in cervical cancer. Such preliminary studies may form the basis for important laboratory aids to the diagnosis of the disease.

Review of Literature

II REVIEW OF LITERATURE

A review of literature pertaining to the study, 'Biochemical and Hormone Profiles in Cervical Cancer' is discussed under the following headings.

1. Classification
2. Incidence
3. Risk Factors Associated with Cervical Cancer
   a) Infection - Herpes simplex virus and cervical cancer
   b) Age at first coitus and sexual behaviour
   c) Age incidence
   d) Use of contraceptives
4. Symptoms
   a) Bleeding
   b) Leu Corrhea
   c) Pain
   d) Urinary symptoms
5. Biochemical Changes
   a) Proteins
   b) Glycoproteins
   c) Fat
   d) Vitamins
   e) Trace elements
   f) Hormones

Cervical cancer remains the most common carcinoma in the genital canal, accounting for approximately 50 percent of all primary malignancy in the pelvis (Novak and Wood-ruff, 1979). When recognised early and successfully treated, it is possible to decrease significantly the frequency of cervical cancer (Stallworthy and Bourne, 1979).

i. Classification

The international classification of the stages of cancer of cervix is as follows.

Pre Invasive Carcinoma

Stage - 0

Includes carcinoma in situ, intraepithelial carcinoma.

Invasive Carcinoma

Stage - I

Carcinoma is strictly confined to the cervix.

Stage - I-a

The cancer cannot be diagnosed by clinical examination. It includes early stromal invasion and occult cancer.

Stage - I-b

This includes all other stages of stage I.

Stage II

The carcinoma extends beyond the cervix but has not extended on to the pelvic wall. The carcinoma involves the vagina but not the lower third.

Stage II-a

No obvious parametrial involvement is noticed.

Stage II-b

In this stage, obvious parametrial involvement is noticed.

Stage III

The carcinoma has extended on to the pelvic wall. On rectal examination there is no cancer free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina.

Stage III-a

There is no extension on to the pelvic wall.

Stage III-b

Extension on to the pelvic wall is observed.

Stage IV

The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum (Kissman, 1965).

PLATE-1

Clinical appearance of epidermoid carcinoma of cervix in early (above) and moderately advanced (below) stages.

2. Incidence

Carcinoma of the uterine cervix is the commonest malignancy seen among women in India (Singh et al., 1983).

It accounted for 20-50 percent of all neoplasms of the body and 80 percent of all malignancies of the female genital tract. A high percentage of cervical carcinoma, that is 25.4, 25.6, 24.4, 30.8, 31.7 and 45.3 percent respectively have been reported from Nepal, Bangladesh, Burma, Thailand, Bangkok and Sri Lanka. In India, the rate of cervical dysplasia has been found to vary between 24-63 per 1000 women in contrast as reported from west. Thus incidence of cervical cancer in Indian women is the highest of all cancers (Luthra et al., 1983).

Two hundred and seventy cases of cancer of cervix were registered in Amala Cancer Hospital and Research Centre - Trichur from November 1981 to January 1985 (Sudheeran et al., 1986).

The incidence rate of cervical cancer in Kuppuswamy Naidu Memorial Hospital, Coimbatore in 1986 was found to be 11.96 percent.

Unhealthy cervix was associated with higher incidence of cervical malignancy. A high incidence of 23.4 percent unhealthy cervix was found at the gynaecology out patient department of SCB Medical College Hospital, Cuttack from November 1978 to June 1981 (Das et al., 1984).

Cervical cancer used to be seen in women of child bearing age, but in the past 5 years, 80 percent of clinical cases have been in post-menopausal patients (Elizabeth and Teper et al., 1978).

There was a small decrease in deaths from carcinoma of the cervix in England and Wales between 1970 and 1976 (Robert, 1978).

The incidence rate of carcinoma of corpus uteri in Japan was approximately one - tenth that of carcinoma of uterine cervix (Yajima et al., 1965).

In the USA, in contrast to the remarkable increase in the numbers of women treated for carcinoma - in - situ of the cervix, the number of patients with invasive cervical carcinoma had decreased to 16,000 in 1980 from 19,000 in 1975 and 20,000 in 1970. After several decades, as the most common gynaecological cancer, cervix cancer is now encountered less often than endometerial cancer which has increased to first place (Kissang, 1985).

3. Risk Factors Associated with Cervical Cancer

a) Infection

Case control study by Dr. Buckley and colleagues suggested that cervical cancer had an infectious aetiology. Some cancers of the penis and cervix may share a common

aetiology, had been suggested by the observation that the wives of men with cancer of the penis experienced an excess of the cancer of cervix (Gardham and Crombie, 1961).

Cancer of the cervix was more frequent in lower social class groups and here hygienic factors play a role (Barnes, 1980).

Herpes virus and cancer of cervix

Herpes simplex virus - 2 (HSV - 2) had been implicated as a potential agent in cervical carcinogenesis. Induction of chromosomal damage by HSV was first reported by Hamper and Rilliam in 1961 (Murthy et al., 1986).

According to Nahmick (1974) women with HSV - 2 antibodies were about 4.5 times more likely to develop cancer of cervix than are women without antibodies to this virus. HSV infection increased the susceptibility of cervical cells to undergo dysplasia changes (Nasani, 1982).

b. Age at first coitus and sexual behaviour

A number of studies have shown that early age at first sexual intercourse to be a risk factor for cervical cancer. Some reports suggested that the young cervix was more susceptible than older cervix to carcinogenic effect of whatever sexually transmitted agent causes cervical cancer (Thomas, 1986).

Young age of first pregnancy also was more significant for cervical cancer incidence than the number of pregnancies (Hassani, 1982).

c. Age incidence

The incidence of cervical cancer increased with age and reached a peak among women aged 55-64. The most at risk of developing cervical cancer were women aged over 35 and older women were most vulnerable to developing the disease (Hobbs et al., 1985).

d. Use of contraceptives

A relative risk for cervical cancer was observed in women who had used depot - medroxy progesterone acetate (DMPA) for more than 5 years. DMPA is a long acting steroid preparation used as a contraceptive (Thomas et al., 1985).

Debaussens and associates concluded the risk of cervical carcinoma in women using long acting progestogens was not significantly different from those using intra-uterine devices (IUD) (Gopalakrishnan and Virkar, 1980).

4. Symptoms

a. Bleeding

Intermenstrual bleeding is a common symptom. This bleeding may be increased by contact after coitus, douching or

insertion of a diaphragm. Later, severe bleeding occurs and the patient becomes anemic.

b. Leucorrhea

Chief symptom is a watery, often offensive blood tinged or blood stained discharge. This may be present for several months and may have a foul odour.

c. Pain

Pain is a late symptom but once it occurs, it may become severe and intractable, radiating from pelvis and lower back into the legs.

d. Urinary symptoms

Urinary symptoms may take the form of frequency, a vesico-vaginal fistula may develop and uraemia may occur (Barnes, 1960).

5. Biochemical Changes

a. Proteins

Total serum protein levels did not differ in cervical cancer patients though the serum albumin values were lowered (< 3.0 g/dl). Serum globulin values were raised and the values paralleled the advancement of the disease. This is indicative of non specific infection.

There was a significant rise in serum IgG levels in cancer cervix patients. The values ranged from 1000 to 2100 mg percent with a mean 156.87 percent ± 263.18. The value of serum IGA did not differ significantly. The rise in IgG with the advancing stages of the disease was partly attributed to the local infection (Sharma et al, 1979).

b. Glycoproteins

Serum glycoproteins were significantly increased in cervical carcinoma. The increase was speculated as a possible factor for the appearance of extra glycoprotein bands in the electrophoresis of serum samples (Balasubramaniam and Govindarajulu, 1977).

A linear increase of serum sialic acid with progression of a malignant growth in cervical carcinoma patients had been reported. Serum sialic acid was elevated in cancer of cervix (3.8 μmol/ml) as compared to matched control group of normal women (1.8 ± 0.25 μmol/ml) (Maity et al., 1983).

These acidic sugars (sialic acid) were secreted in large quantities bound to glycoproteins eg: serum and mucus glycoproteins or to oligosaccharides of urine and milk. Not much is known either about the regulation of sialic acid biosynthesis (by hormones) or the reasons for increased sialylation and sialyl transferase activity observed in cancer cells (Schauer, 1985).

c. Fat

In women, 38-70 years old, with carcinoma of cervix, HDL cholesterol in plasma (44 mg/100 ml) was significantly lower than values in healthy controls. Total cholesterol (266 mg/100 ml) was significantly greater (Andisio et al., 1986).

Non esterified cholesterol hypersecretion in urine occured with active carcinoma of cervix including carcinoma in situ (Acevedo, 1975).

d. Vitamins

Vitamin assessment was made in 78 patients with untreated cancer of uterine cervix. Ascorbic acid was low in 67 percent of patients. Plasma folate and retinol were also significantly low (Orr et al., 1985).

The mean concentration of ascorbic acid in the plasma was significantly lower in the cases than in the controls i.e. 0.36 against 0.75 mg/dl (Romney, 1986).

e. Trace elements

Forty five percent of patients had an elevated level of serum mean cu/zn ratio and patients with advanced disease were more likely to have a higher cu/zn ratio. Serum iron showed a stage related decrease (Orr et al., 1985).

Patients with untreated cervical cancer had high serum ferritin values. Upper limit of ferritin for controls

was 108 ng/ml. Ferritin values decreased to the normal range four weeks after treatment in many patients (Takagi et al., 1981).

The serum selenium in patients seemed to be mainly dependant on dietary factors. The mean serum selenium concentration in patients with cervix cancer increased from the year 1973-1982 and decreased in 1983 when the amount of selenium rich grain was reduced (Sundstrom, 1986).

f. Hormones

Considering the incidence of cancer in different tissues of the body with respect to male and female, hormones have been indicated to be some of the causes of such disease. There is evidence that prolonged stimulation to cell division acts as one of the factors causing the tissues become carcinomous. The estrogens not only stimulate the growth and differentiation of accessory sex organs but also other tissues of the body (Chatterjee, 1990).

Nohlanns et al (1976) using the indirect peroxidase labeled technique, demonstrated on HCG - like substance in frozen tissue sections of most cancers. Data indicated that cervical squamous cell carcinoma produced hcG - like substance less frequently than other tumors, if anti-hcG antibody is reliable (Du-Zen-Tsai, 1985).

Experimental Procedure

III EXPERIMENTAL PROCEDURE

The experimental procedures relating to the study "Biochemical and Hormone Profiles in Cervical Cancer" are presented in the following sequence.

1. Selection of subjects
2. Collection of blood
3. Separation of serum
4. Separation of plasma
5. Estimation of plasma ascorbic acid
6. Estimation of serum cholesterol
7. Estimation of serum total creatinine
8. Estimation of serum total protein, albumin and globulin.
9. Estimation of serum zinc
10. Estimation of serum copper
11. Estimation of serum iron
12. Estimation of serum manganese
13. Estimation of serum prolactin
14. Estimation of serum estradiol
15. Statistical analysis

1. Selection of subjects:

Sixteen cervical cancer patients undergoing treatment in Kuppuswamy Naidu Memorial Hospital and five similar patients in Government Hospital, Coimbatore participated

for the present study. Except two, all the others were in-patients and belonged to the age group 26-67 years.

Nine normal healthy individuals of the same age group were studied as controls for comparision.

2. Collection of blood:

The blood was collected as follows (Omer - 1976). Tied a tourniquiet (of soft rubber tubing or a strip of bandage) tightly around the arm of the patient, a couple of inches above the elbow. Had the subject clench his fist firmly, washed the skin surface about the prominent vein on the inner surface of the elbow with 70 percent alcohol allowed to dry, held the vein immobile by pressing on it with the thumb below the elbow and into the vein inserted a sharp sterile hypodermic needle an inch to a half long which was attached to a dry sterile syringe of suitable capacity. The needle should penetrate the vein from the outside at an angle of 30° with the surface of the arm, the level or opening of the needle being kept upward or to the side. As soon as the blood was seen to enter the syringe, retracted the plunger slowly until the desired amount of blood had entered the syringe. Before removing needle from the vein, loosened the tourniquiet, had the patient unclench his fist and on the skin, at the point of entrance of the needle, held in place a small pad

of folded gauze moistened with 70 percent alcohol, withdrew the needle, detached it from the syringe and then transferred the blood to a centrifuge tube, pressure on the gauze pad will effectively prevent bleeding from the skin puncture. It is important that the pressure be maintained for a minimum of 5 minutes, to prevent the formation of a painful hematoma at the site of puncture.

3. Separation of serum:

The blood after being transferred to a centrifuge tube, was allowed to clot. The clot was removed and centrifuged after which the supernatant was separated. The separated serum was frozen till used for analysis.

4. Separation of plasma:

An anticoagulant, potassium oxalate was placed into the tube into which blood was collected. The plasma was removed from the cells soon after collection (Tietz - 1976).

5. Estimation of plasma ascorbic acid

Plasma ascorbic acid was estimated by "Roe and Kuether" method using 2, 4 dinitro phenyl hydrazine (Varley et al.,1966).

The details are presented in Appendix I.

6. Estimation of serum cholesterol

Serum cholesterol was estimated by Zak's method (Varley, 1975) the details of which are presented in Appendix II.

7. Estimation of serum total creatinine:

The amount of creatinine in serum was estimated by "Alkaline Picrate Method" (Varley et al., 1980) the details of which are given in Appendix III.

8. Estimation of serum total protein, albumin and globulin:

The total protein, albumin and globulin level in serum was estimated by 'Biuret Method' (Varley et al., 1980).

The details are given in Appendix IV.

9. Estimation of serum zinc:

The serum zinc level was estimated by "Piper's Method" (1968) in Atomic Absorption Spectrophotometer.

The details of the method is given in Appendix V.

10. Estimation of serum copper:

The serum copper level was estimated by "Piper's Method" (1968) in Atomic Absorption Spectrophotometer, the details of which are presented in Appendix V.

11. Estimation of serum iron:

The amount of iron in serum was estimated by "Piper's Method" (1969) in Atomic Absorption Spectrophotometer, the details of which are given in Appendix V.

12. Estimation of serum manganese:

The serum content of manganese was estimated by "Piper's Method" (1969) in Atomic Absorption Spectrophotometer. The details of the method is given in Appendix V.

13. Estimation of serum prolactin:

The amount of prolactin in serum was estimated by "Radio Immuno Assay" (Diagnostic Products Corporation (DPC) - 1985). The details of the method is given in Appendix VI.

14. Estimation of serum estradiol:

The amount of estradiol in serum was estimated by "Radio Immuno Assay" (Diagnostic Products Corporation (DPC) - 1985) the details of which are presented in Appendix VII.

15. Statistical analysis:

't' tests were conducted wherever necessary to check if the results were significant using the formula:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2} \times \frac{1}{n_1} + \frac{1}{n_2}}}$$

where $\bar{X}_1$ = mean of sample - 1

$\bar{X}_2$ = mean of sample - 2

n_1 = number of observations in sample - 1

n_2 = number of observations in sample - 2

s_1 = Standard deviation of sample - 1

s_2 = Standard deviation of sample - 2

If the number of observations in sample -1 and 2 (n_1 and n_2) are equal, then the formula used was:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2 + s_2^2}{n-1}}}$$

where

$\bar{X}_1$ = mean of sample - 1

$\bar{X}^2$ = mean of sample - 2

s = standard deviation of sample - 1

s_2^1 = standard deviation of sample - 2

Results and Discussion

IV RESULTS AND DISCUSSION

The results pertaining to the study on "Biochemical and Hormone Profiles in Cervical Cancer are discussed in this chapter.

Patients with cervical cancer were studied. They were distributed under the I, II and III stages of cervical cancer depending on the severity of the condition. Their serum and plasma were analysed for biochemical and hormonal changes. Ascorbic acid in plasma and cholesterol, total creatinine, total protein, albumin, globulin, zinc, copper, iron, manganese, prolactin and estradiol in serum were estimated. The hormones, prolactin and estradiol were estimated by Radio Immuno Assay. The values obtained were compared with those of normal individuals who served as controls to see whether there was any significant change in serum and plasma levels of the above mentioned parameters. The main aim of the above study was to find out any possible biochemical marker which may be useful in the earlier diagnosis of cervical cancer.

1. Distribution of the target cervical cancer patients among different stages:

Sixteen patients undergoing treatment in Kuppuswamy Naidu Memorial Hospital and five similar patients in Government Hospital, Coimbatore participated for the present

study. These patients could be divided in three different stages of cervical cancer namely stage I, stage II and stage III*. The distribution of patients according to the different stages of cervical cancer are presented in Table I.

TABLE I

DISTRIBUTION OF THE TARGET CERVICAL CANCER PATIENTS AMONG DIFFERENT STAGES

Patients	Stages of Cervical Cancer			Total
	Stage I	Stage II	Stage III	
Number	5	7	9	21
Age in Years	45-67	26-51	40-65	

The women patients were 26 to 67 years age. Nine apparently normal healthy women of the same age group were subjected to similar investigations for comparison. These women served as positive control groups.

* - The scientific basis for the classification of stages I, II and III of cervical cancer is discussed in pages 7&8, Chapter II - Review of literature.

2. Biochemical and hormonal changes

a. Changes in plasma ascorbic acid

Low dietary intake of ascorbic acid by women with cervical cancer, significantly decreased the plasma ascorbic acid concentration as reported by Romney et al.,(1955).

Table II presents the mean plasma ascorbic acid levels in normal individuals and patients with different stages of cervical cancer. Figure I depicts the same diagramatically.

It was seen from Table II that the plasma ascorbic acid was decreased in cervical cancer patients (0.4 mg/dl) compared to normal (1.4 mg/dl). The decrease was significant at 1 percent level in all the three stages, but no significant difference was noted between the different stages. The severity of cervical cancer did not make any further change in plasma ascorbic acid level.

The above results were in agreement with those of Romney et al., (1955) who reported that the plasma ascorbic acid was significantly lower in patients with cervical cancer (0.36 mg/dl) compared to controls (0.75 mg/dl).

Thus plasma ascorbic acid may be used as an indicator in diagnosis of cervical cancer. In the present study, the dietary intake of ascorbic acid were not known.

TABLE II

PLASMA ASCORBIC ACID IN mg/dl

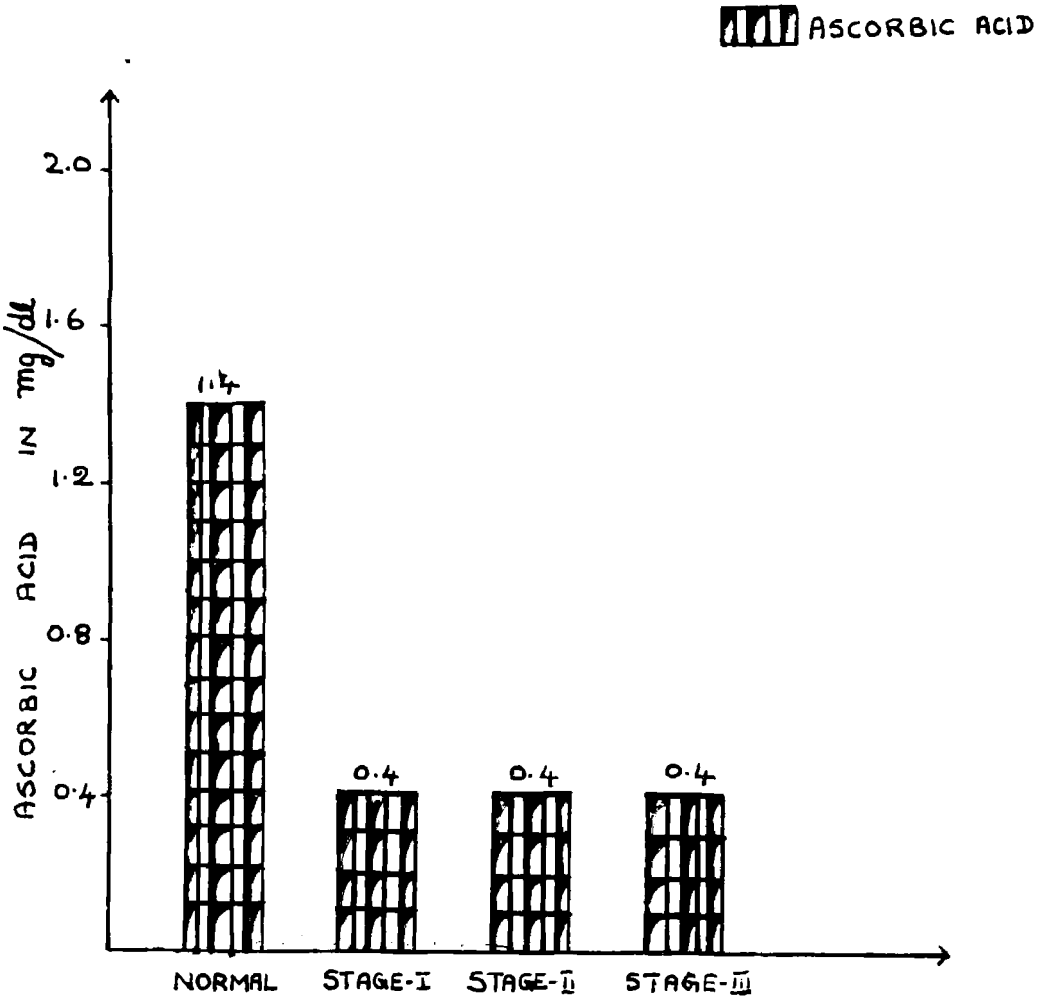
Experimental samples	Ascorbic Acid Mean ± SD mg/dl	Groups Compared	Statistical Significance
Normal	1.4 ± 0.25		
(a)	(a_1)	a_1 V$_s$ b_1	7.95**
		a_1 V$_s$ c_1	9.33**
Cervical Cancer			
Stage I	0.4 ± 0.1	a_1 V$_s$ d_1	10.9**
(b)	(b_1)	b_1 V$_s$ c_1	NS
Stage II	0.4 ± 0.1	b_1 V$_s$ d_1	NS
(c)	(c_1)	c_1 V$_s$ d_1	NS
Stage III	0.4 ± 0.07		
(d)	(d_1)		

** - Significant at 1 percent level

NS - Not Significant

FIG-I

PLASMA ASCORBIC ACID IN DIFFERENT STAGES OF CERVICAL CANCER



b. Changes in serum cholesterol

Table III and figure II presents the serum cholesterol levels in normal individuals and cervical cancer patients of different stages.

In cervical cancer, serum cholesterol levels were increased significantly at 1 percent level when compared to normal subjects (188 mg/dl). No significant differences were noticed between stages I (250 mg/dl) and II (258 mg/dl) and stages I and III (246 mg/dl) whereas between stages II and III there was a significant decrease at 5 percent level.

Andisio et al.,(1986) reported serum cholesterol value of 256 mg/dl in cervical cancer patients which was higher compared to controls.

Fat being a sole factor in carcinogenesis, it is essential to restrict fat intake (Bansal, 1986).

Marshall et al., (1984) reported that the index of fat consumption was positively associated with the risk of developing cervical cancer. The increase in serum cholesterol may be due to administration of steroid drugs, but the exact reason for the increase in serum cholesterol is not known.

Serum cholesterol levels may thus aid in diagnosis of cervical cancer.

TABLE III

SERUM CHOLESTEROL IN mg/dl

Experimental Samples	Cholesterol Mean ± SD	Groups Compared	Statistical Significance
Normal (a)	188 ± 15.6 (a_1)	a_1 Vs b_1	7.57**
Cervical Cancer			
Stage I (b)	250 ± 9.36 (b_1)	a_1 Vs c_1	22.17**
		a_1 Vs d_1	7.97**
Stage II (c)	258 ± 6.9 (c_1)	b_1 Vs c_1	1.55 NS
Stage III (d)	245 ± 13.0 (d_1)	b_1 Vs d_1	0.703 NS
		c1 vs d1	2.24*

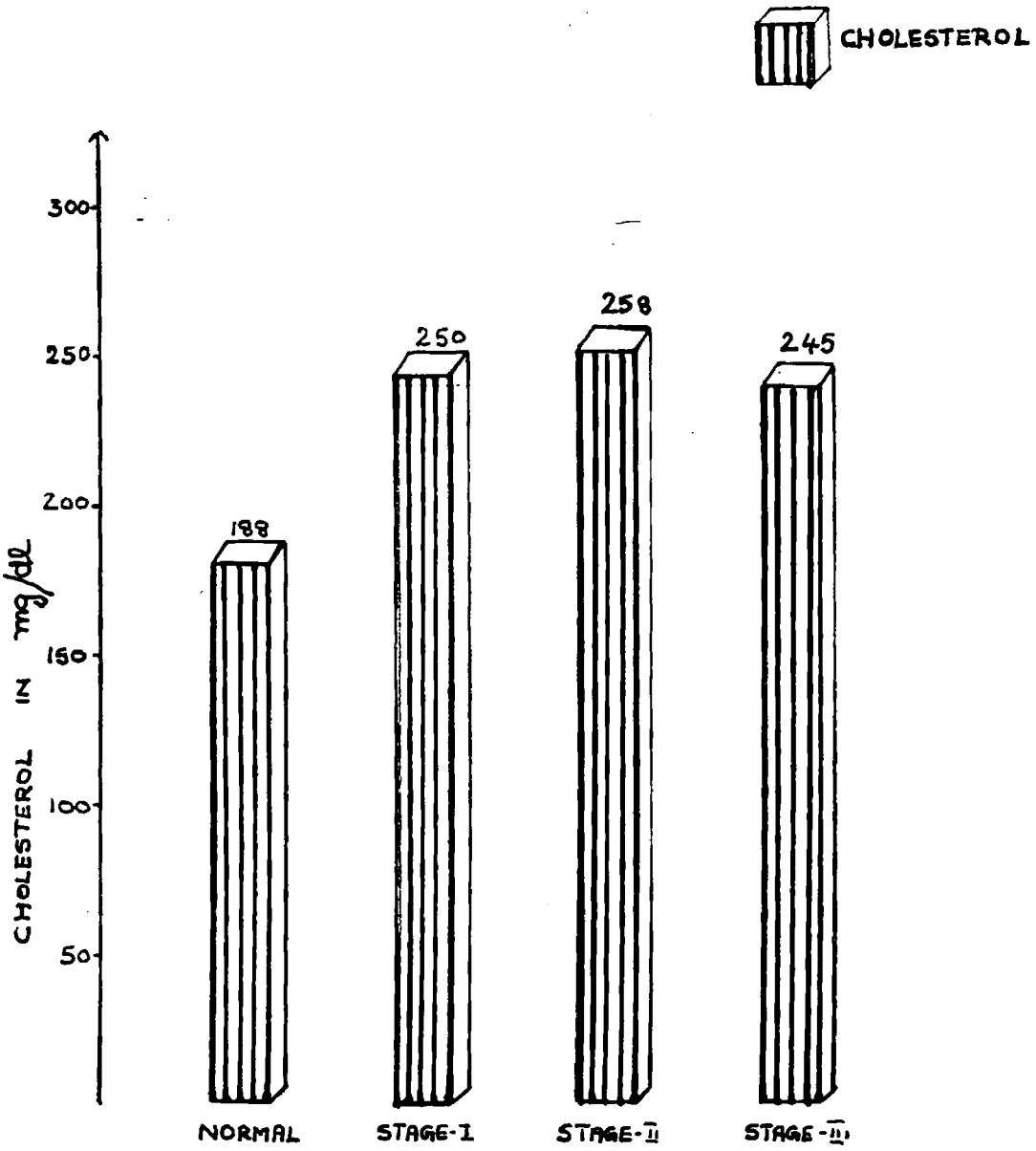
* Significant at 5 percent level

** Significant at 1 percent level

NS Not Significant

FIG-II

SERUM CHOLESTEROL IN DIFFERENT STAGES OF CERVICAL CANCER



e. Changes in serum total creatinine

The changes in serum total creatinine of normal individuals and cervical cancer patients are presented in Table IV. Figure III indicates the same diagrammatically.

In the present study, the serum total creatinine was found to be increased in patients with cervical cancer compared to normal individuals (1.0 mg/dl). The increase was significant at 5 percent level in stage II (1.6 mg/dl) and at 1 percent level in stages I (1.82 mg/dl) and III (1.75 mg/dl). There was no significant change between the different stages of cervical cancer.

The increase may be probably due to increased muscle break down and uraemia noticed in the cases of cervical cancer patients. Thus there may be one or more reasons for the increase in serum total creatinine.

Serum total creatinine may hence serve as a tool for diagnosis of cervical cancer.

TABLE IV

SERUM TOTAL CREATININE IN mg/dl

Experimental Samples	Total Creatinine Mean ± S.D.	Groups Compared	Statistical Significance
Normal	1.0 ± 0.3		
(a)	(a_1)	a_1 Vs b_1	4.32**
Cervical Cancer			
Stage I	1.82 ± 0.33	a_1 Vs c_1	2.78*
(b)	(b_1)	a_1 Vs d_1	3.86**
Stage II	1.6 ± 0.5	b_1 Vs c_1	0.735 NS
(c)	(c_1)	b_1 Vs d_1	0.278 NS
Stage III	1.75 ± 0.46	c_1 Vs d_1	0.58 NS
(d)	(d_1)		

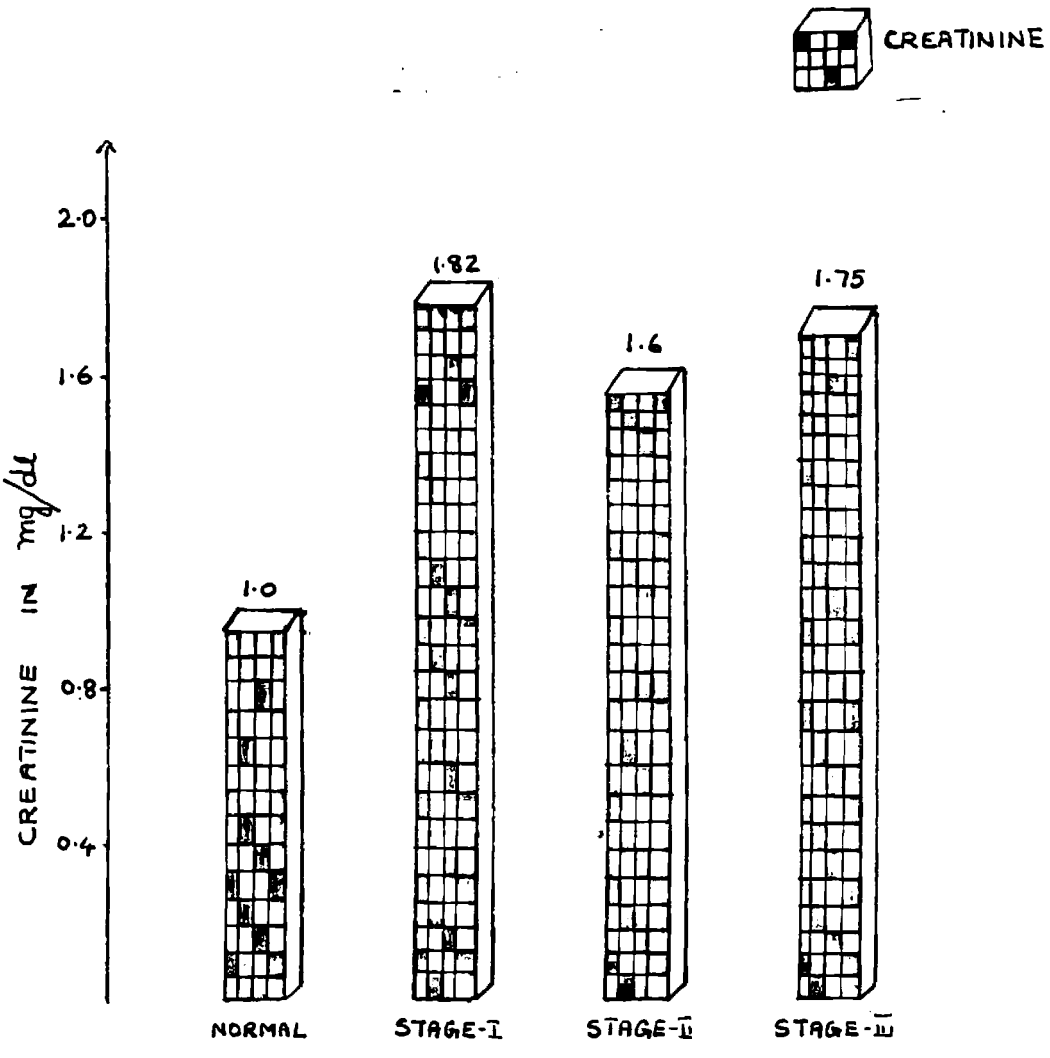
* - Significant at 5 percent level

** - Significant at 1 percent level

NS - Not Significant

FIG- III

SERUM TOTAL CREATININE IN DIFFERENT STAGES OF CERVICAL CANCER



d. Changes in serum total protein, albumin and globulin

Table V and figure IV depicts the levels of serum total protein, albumin and globulin in normal individuals and patients with cervical cancer.

Total protein

Total serum protein levels increased significantly at 1 percent level in different stages of cervical cancer (8.4 g/dl) compared to normal women (7.02 g/dl). No significant differences were noticed between the different stages.

The above results were in contrast to those of Sharma et al., (1979) who reported that the serum total protein levels did not differ significantly in cervical cancer patients.

Albumin

It was seen from Table V and figure IV that the serum albumin level was decreased in the different stages of cervical cancer at 1 percent level when compared to normal subjects (4.2 g/dl). There was significant decrease noticed between stages I (3.5 g/dl) and III (3.2 g/dl) at 5 percent level.

The results thus obtained were in agreement with those of Sharma et al (1979) who reported a lower value of 3 g/dl when compared to normal (4 g/dl).

TABLE V

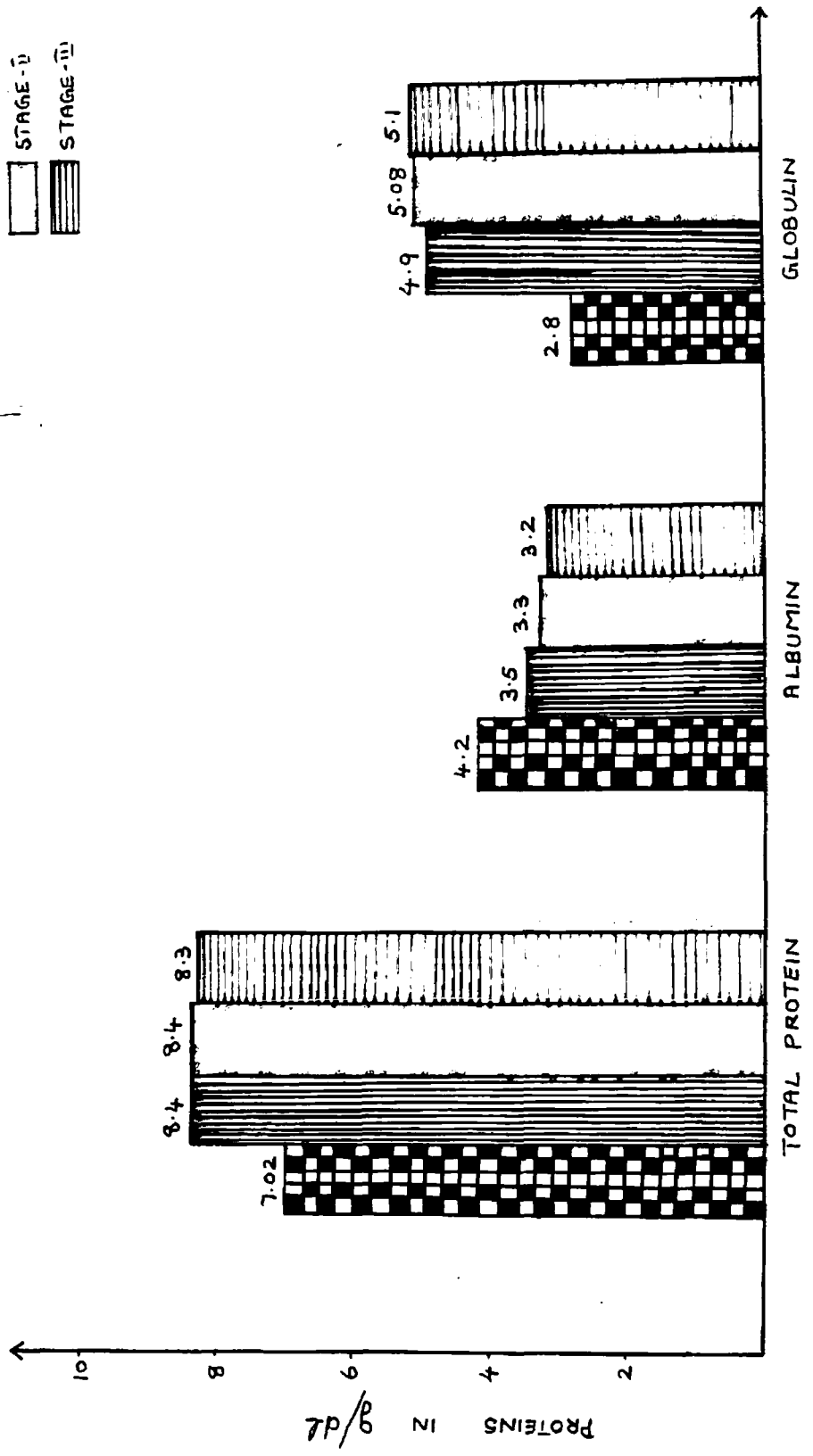
SERUM TOTAL PROTEIN, ALBUMIN AND GLOBULIN IN g/dl

Experimental Samples	Mean ± S.D.			Groups Compared	Statistical Significance
	Total Protein	Albumin	Globulin		
Normal	7.08± 0.2	4.2 ± 0.34	2.82 ± 0.4	a_1 v$_s$ b_1	9.6**
				a_1 v$_s$ c_1	7.51**
(a)	(a_1)	(a_2)	(a_3)	a_1 v$_s$ d_1	7.89**
				b_1 v$_s$ c_1	0 NS
				b_1 v$_s$ d_1	0.46 NS
				c_1 v$_s$ d_1	0.49 NS
Cervical Cancer					
Stage I	8.4± 0.26	3.5 ± 0.16	4.9 ± 0.2	a_2 v$_s$ b_2	4.03**
				a_2 v$_s$ c_2	5.81**
(b)	(b_1)	(b_2)	(b_3)	a_2 v$_s$ d_2	6.7**
				b_2 v$_s$ c_2	1.69 NS
				b_2 v$_s$ d_2	2.24*
				c_2 v$_s$ d_2	0.8 NS
Stage II	8.4±0.43	3.3 ± 0.2	5.08 ± 0.27	a_3 v$_s$ b_3	10.1**
				a_3 v$_s$ c_3	12.02**
(c)	(c_1)	(c_2)	(c_3)	a_3 v$_s$ d_3	12.44**
Stage III	8.3 ± 0.4	3.2 ± 0.25	5.1 ± 0.33	b_3 v$_s$ c_3	1.15 NS
				b_3 v$_s$ d_3	1.14 NS
(d)	(d_1)	(d_2)	(d_3)	c_3 v$_s$ d_3	0.12 NS

* Significant at 5 percent level ** Significant at 1 percent level
NS Not Significant

FIG-IV

SERUM TOTAL PROTEIN, ALBUMIN AND GLOBULIN
IN DIFFERENT STAGES OF CERVICAL CANCER



Globulin

In cervical cancer, serum globulin level was found to be increased (5.0 g/dl) when compared to normal (2.82 g/dl) and this increase was significant at 1 percent level. But there was no significant difference noticed between the different stages of cervical cancer.

These values agreed with those of Sharma et al., (1979) who reported an increased serum globulin level in cervical cancer patients (3.9 g/dl) compared to normal women (2.9 g/dl).

The above results indicate that the serum total protein, albumin and globulin levels may serve as an indicator for diagnosis of the disease.

e. Changes in serum zinc

Studies suggested that dietary zinc was associated with an increase in the incidence of cancer at certain sites. Serum zinc levels in such cases were found to be lower compared to controls.

The serum zinc level in normal individuals and cervical cancer patients are presented in Table VI and figure V.

In the present study, there was a significant decrease in serum zinc level in stage III (77.6 µg/dl) when compared to normal (98.5 µg/dl) and stage I (136.6 µg/dl). The decrease was significant at 5 percent level.

The decrease may be due the debilitating disorder or renal failure noticed in cervical cancer. Prasad (1985) suggested that this trace element zinc may have a role in regulation of prolactin release. Treatment with zinc lowered serum prolactin level.

The results were in agreement with Orr et al., (1985) who reported a decreased serum zinc level in cervical cancer patients (84 µg/dl) against control women.

f. Changes in serum copper

Table VI and figure V depicts the serum copper levels in normal individuals and cervical cancer patients in different stages.

In this study, it was seen that there was a significant increase in serum copper level in stage II (153 µg/dl) at 5 percent level and in stage III (20 µg/dl) at 1 percent level when compared to normal (114.7 µg/dl). The increase paralleled the advancement of the disease and it is in the order,

Stage I < Stage II < Stage III

The results agreed with those reported by Orr *et al.*, (1985) who reported a value of 160 µg/dl in cervical cancer patients which was significantly higher than in controls. This high levels of copper might have been associated with post menopausal stage, as the levels were found to be low in ovulating females as reported by Pandey *et al.*, (1986).

c. Changes in serum iron

The levels of serum iron in normal individuals and in different stages of cervical cancer are presented in Table VI, Figure V depicts the same diagrammatically.

A significant decrease in serum iron level was noticed in stage III (49.3 µg/dl) when compared to normal (97.5 µg/dl) stage I (108.8 µg/dl) and II (95 µg/dl). The decrease was significant at 1 percent level and this decrease was probably due to excessive bleeding. Bleeding increased with the advancement of the disease.

These values are also in agreement with that reported by Orr *et al.*, (1985) which was found to be 55 µg/dl. This value was significantly lower than normal controls.

Thus the serum iron level may serve as a tool for diagnosis of cervical cancer.

TABLE VI

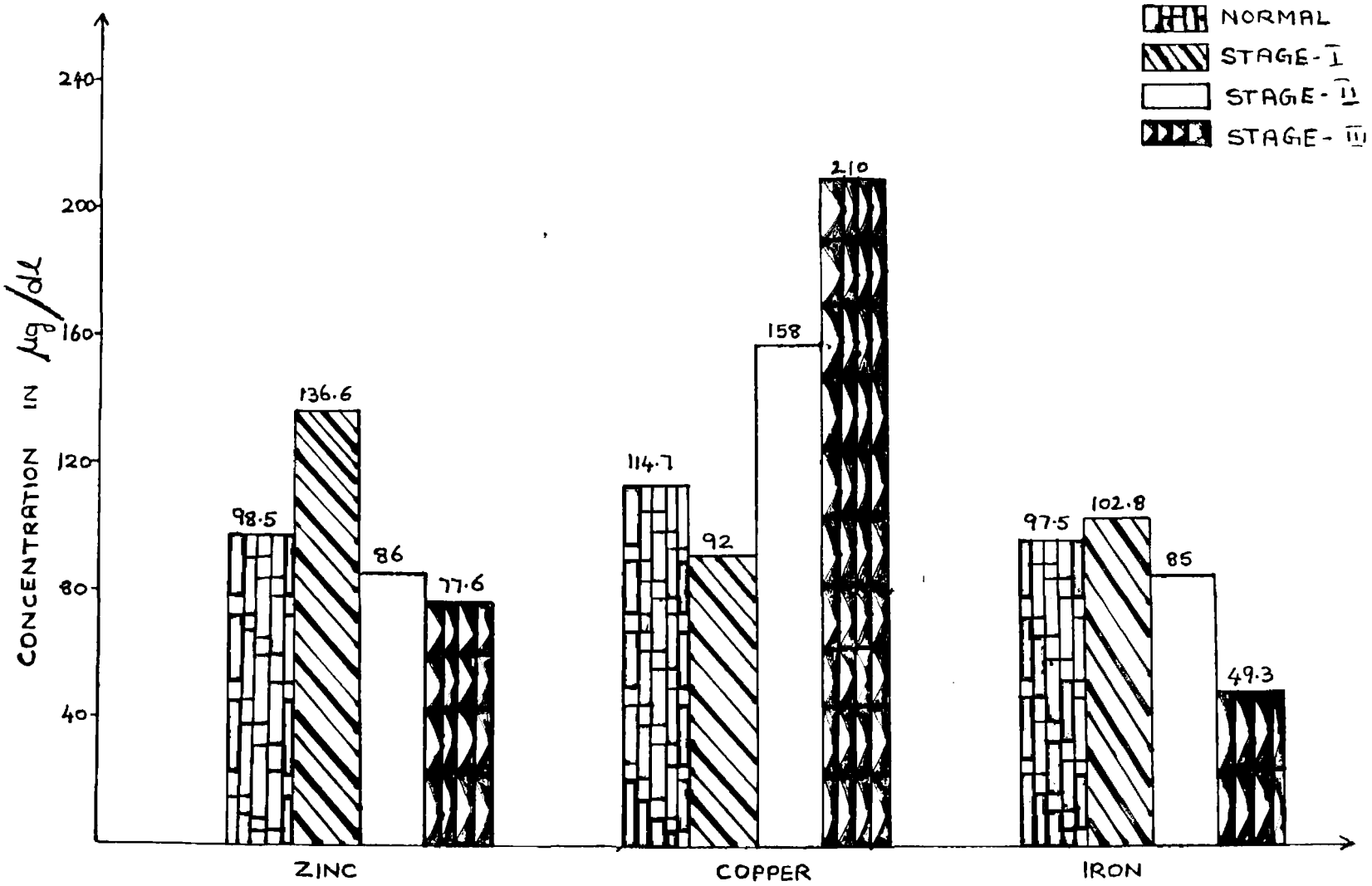
SERUM ZINC, COPPER AND IRON IN µg/dl

Experimental Samples	Mean ± S.D.			Groups Compared	Statistical Significance
	Zinc	Copper	Iron		
Normal	93.6 ± 6.4	114.7 ± 13.7	97.6 ± 15.6	a_1 vs b_1	1.97 NS
				a_1 vs c_1	1.65 NS
				a_1 vs d_1	2.81*
(a)	(a_1)	(a_2)	(a_3)		
				b_1 vs c_1	2.08 NS
				b_1 vs d_1	2.76*
				c_1 vs d_1	0.756 NS
Cervical Cancer				a_2 vs b_2	1.52 NS
Stage I	136.6 ± 53.0	92.0 ± 33.0	102.8 ± 16.0	a_2 vs c_2	2.68*
				a_2 vs d_2	11.45**
(b)	(b_1)	(b_2)	(b_3)		
				b_2 vs c_2	2.76*
				b_2 vs d_2	5.72**
				c_2 vs d_2	2.89*
Stage II	96.0 ± 21.4	153.0 ± 40.0	85.0 ± 17.0	a_3 vs b_3	0.56 NS
				a_3 vs c_3	1.43 NS
(c)	(c_1)	(c_2)	(c_3)	a_3 vs d_3	6.46**
				b_3 vs c_3	1.67 NS
				b_3 vs d_3	5.99**
				c_3 vs d_3	4.26**
Stage III	77.6 ± 25.0	210.0 ± 38.0	49.3 ± 14.3		
(d)	(d_1)	(d_2)	(d_3)		

* Significant at 5 percent level
** Significant at 1 percent level
NS Not Significant

FIG-V

SERUM ZINC, COPPER AND IRON IN DIFFERENT STAGES OF CERVICAL CANCER



h. Changes in serum manganese

Table VII and figure VI indicates the serum manganese levels in both normal individuals and patients with cervical cancer in different stages.

It was seen that the serum manganese level was increased in stage II (1.51 µg/dl) of cervical cancer compared to normal (0.95 µg/dl). This increase was significant at 5 percent level. There was no other significant change with respect to manganese in the different stages of cervical cancer. The increase noted may be due to the post menopausal stage of the patients.

TABLE VII

SERUM MANGANESE IN µg/dl

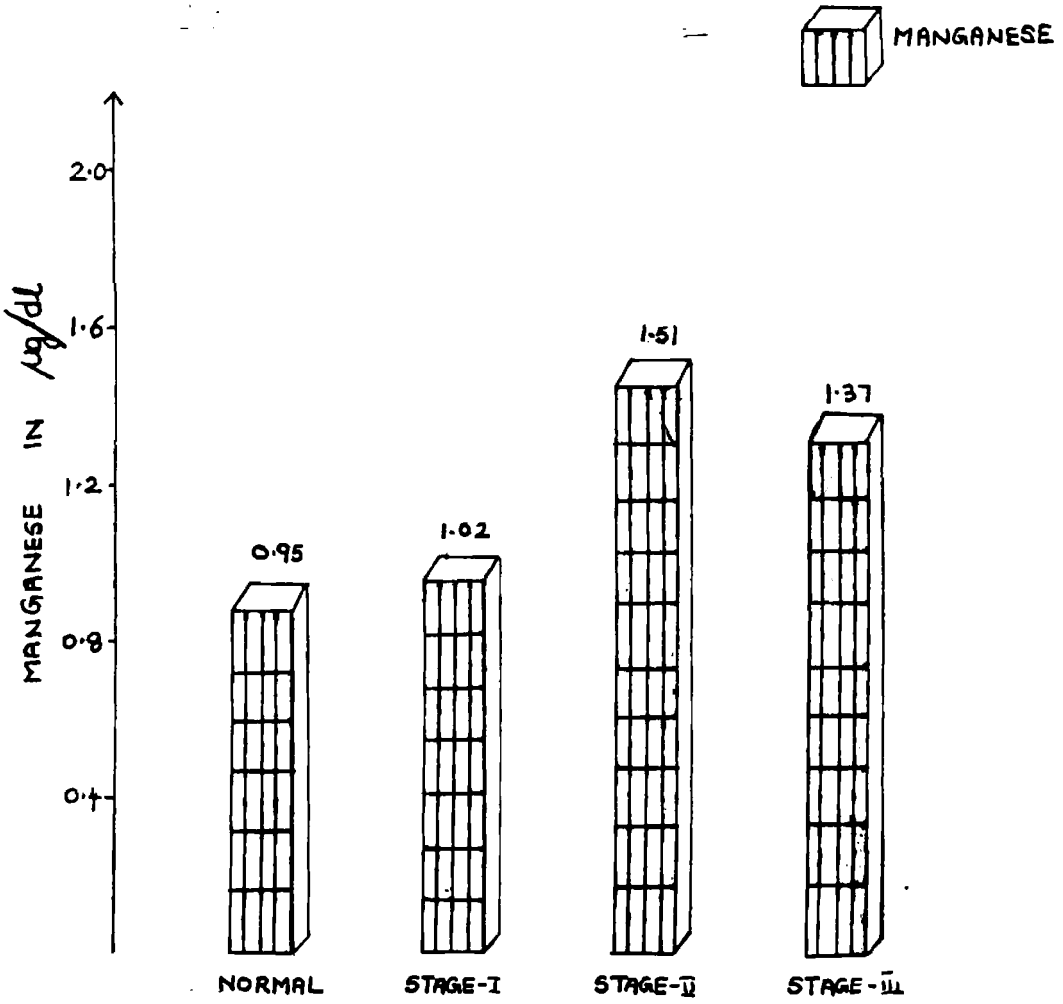
Experimental Samples	Manganese Mean ± SD	Groups compared	Statistical Significance
Normal (a)	0.95 ± 0.30 (a_1)	a_1 Vs b_1	0.39 NS
Cervical Cancer		a_1 Vs c_1	2.7*
Stage I (b)	1.02 ± 0.29 (b_1)	a_1 Vs d_1	1.86 NS
Stage II (c)	1.61 ± 0.47 (c_1)	b_1 Vs c_1	1.88 NS
		b_1 Vs d_1	1.2 NS
Stage III (d)	1.37 ± 0.56 (d_1)	c_1 Vs d_1	0.86 NS

* Significant at 5 percent level

NS . Not Significant

FIG-VI

SERUM MANGANESE IN DIFFERENT STAGES OF CERVICAL CANCER



1. Changes in serum prolactin

Prolactin is the adenohypophysical lactogenic hormone. It has antigonodotropic action in humans. The antigonadal action of prolactin is apparent, for example, in the frequent occurance of amenorrhea in hyperprolactinaemic states. Although the cause is uncertain, several mechanisms may be involved (White and Handler, 1986).

In the present study, the estimation of hormones were carried out by Radio Immuno Assay (RIA). Serum prolactin and estradiol were assayed in 6 patients only, as the procedure was sophisticated and costly. Hence the data could not be analysed for the different stages of cervical cancer as is done for other biochemical parameters.

Table VIII and figure VII depicts the values of serum prolactin in normal individuals and cervical cancer patients.

In the study, the level of serum prolactin was found to be increased (15.9 ng/ml) in cervical cancer patients compared to normal postmenopausal women (15 ng/ml). The normal values were obtained from the pamphlet provided along with the RIA kit.

TABLE VIII

SERUM PROLACTIN (ng/ml) AND ESTRADIOL (pg/ml)

Experimental Samples	Prolactin Mean ± SD	Estradiol Mean ± SD
Normal	15.0	14.0
Cervical Cancer	15.9 ± 19.0	2.0 ± 1.4

FIG-VII

SERUM PROLACTIN IN CERVICAL CANCER

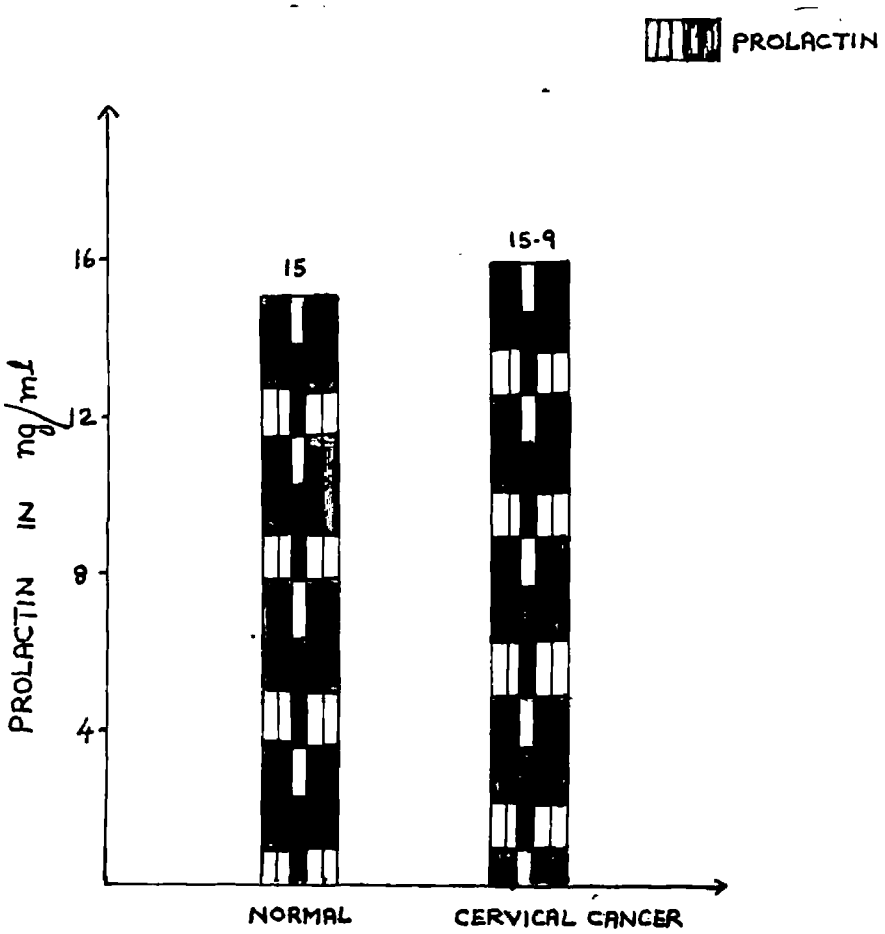
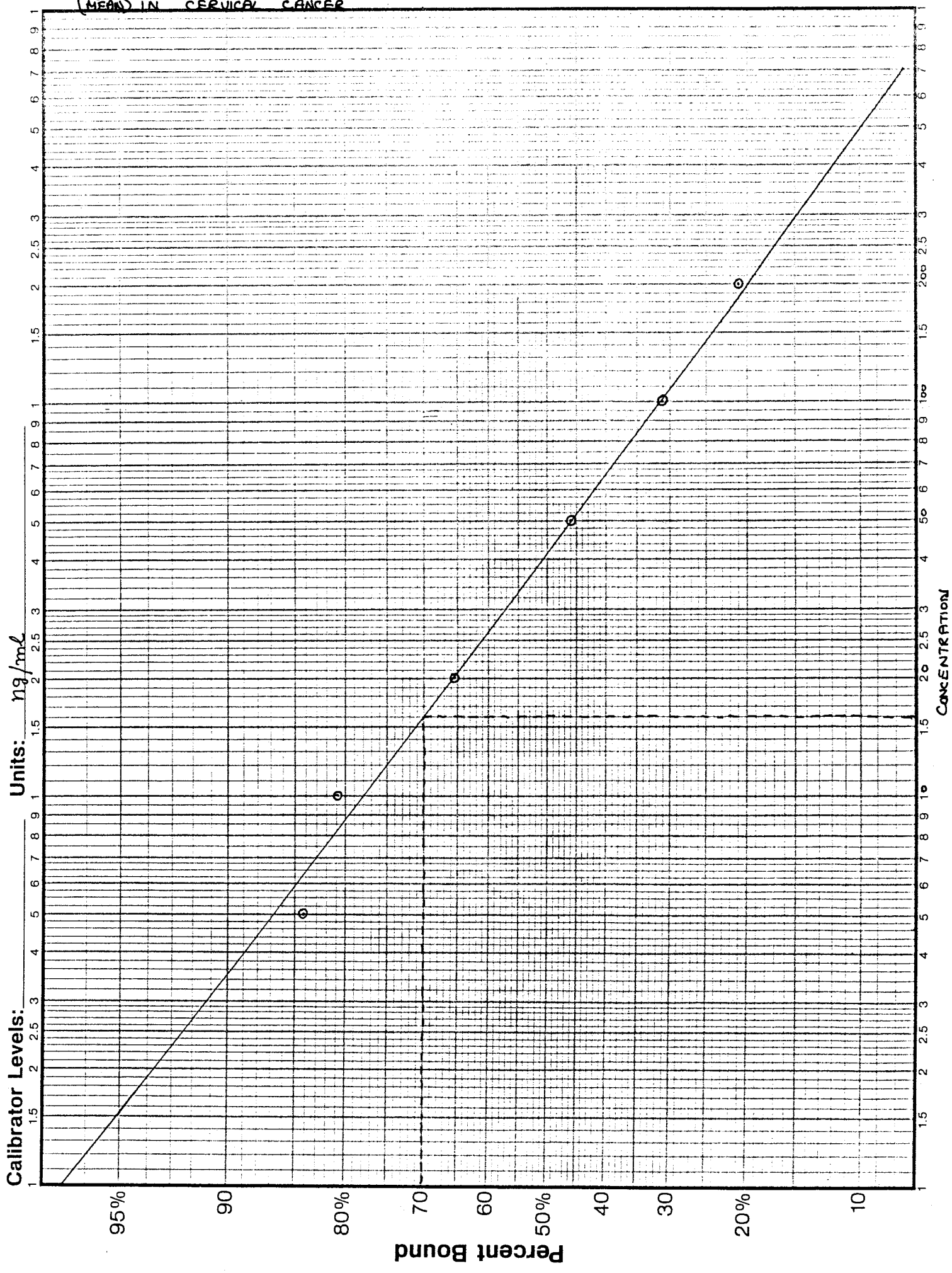


FIG - VIIa

Calibrator Levels: _____ Technician: _____

Analyte: SERUM PROLACTIN (MEAN) IN CERVICAL CANCER Units: _____ Date Run: 5.2.'87



Prolactin hormone levels between 15-100 ng per ml might be due to drug intake or other disorders that interfere with normal hypothalamus inhibition of prolactin secretion. Levels greater than 100 ng/ml almost always indicated a tumor as reported by Zervas and Martin et al., (1980).

Wynder and Rose (1964) reported that high fat diet significantly increased mean prolactin output.

3. Changes in serum estradiol

Serum estradiol levels in normal individuals and cervical cancer patients are presented in Table VIII and figure VIII.

Following menopause, clinical evidence of reduced estrogen production became obvious. Circulating estradiol levels decreased from a mean premenopausal value of 180 pg/ml to 14 pg/ml following cessation of ovarian function (De Fazio and Speroff., 1935).

In the present study, serum estradiol was found to be decreased (8 pg/ml) in cervical cancer patients compared to normal menopausal subjects (14 pg/ml). The reason for the decrease in serum level of estradiol seemed to be inconclusive.

FIG- VIII

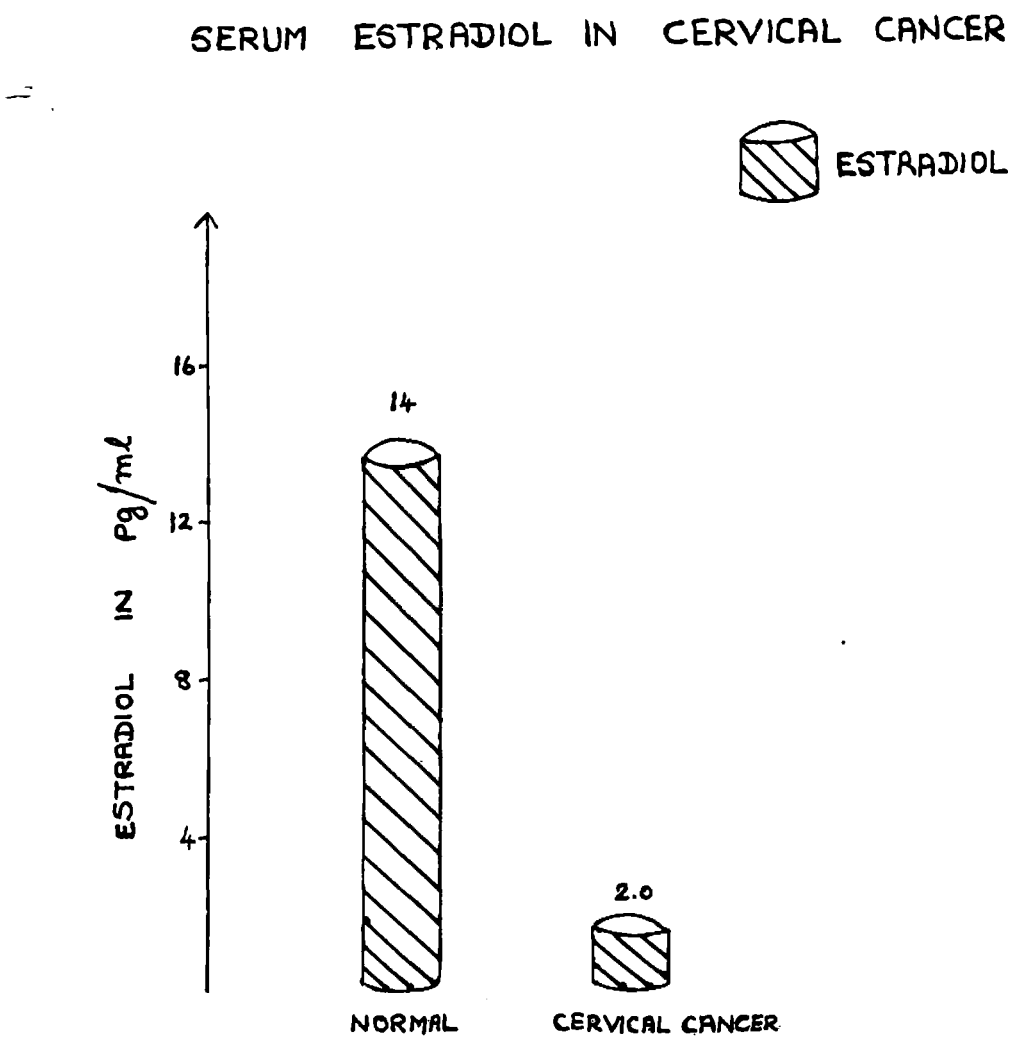
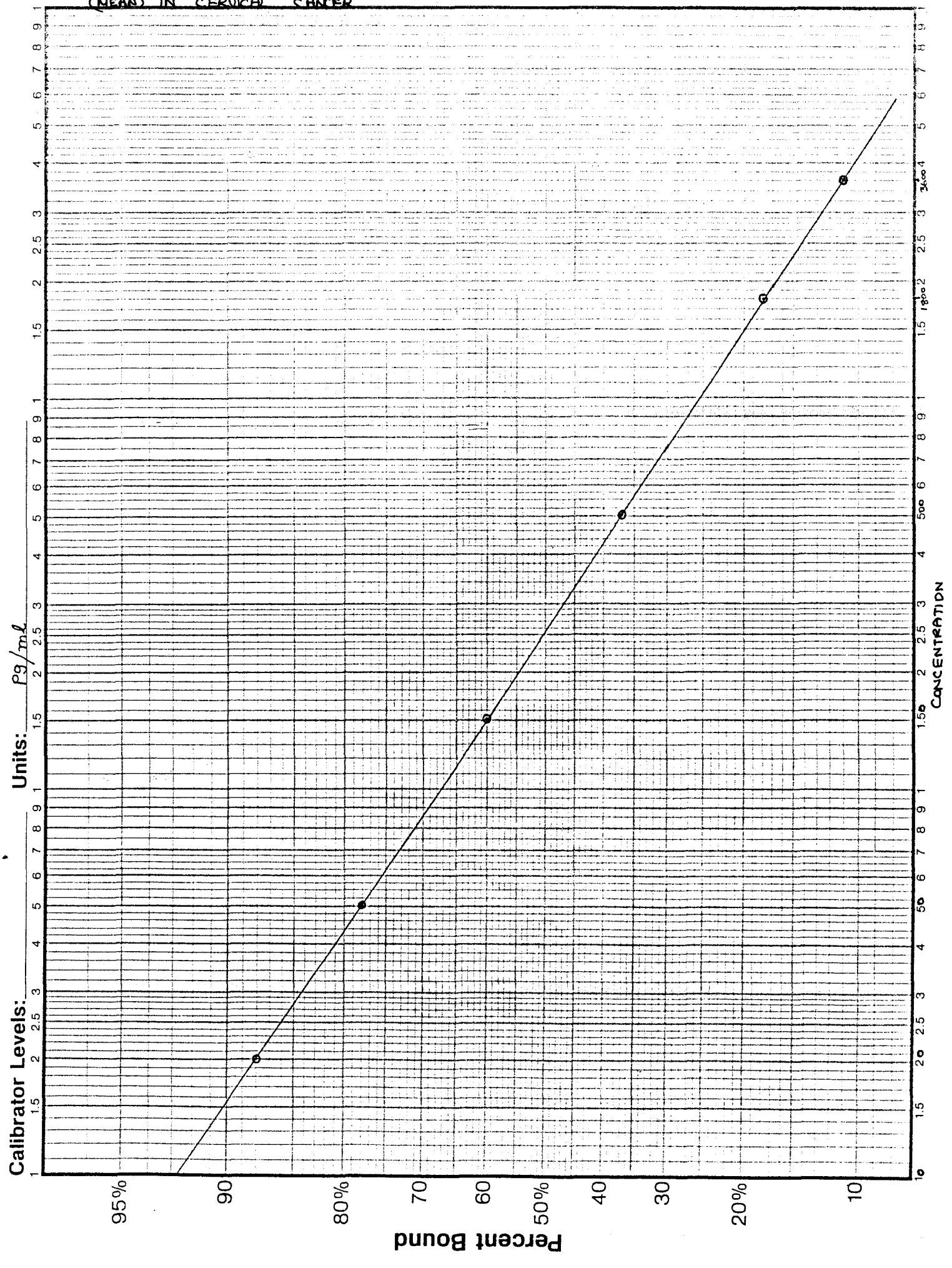

SERUM ESTRADIOL IN CERVICAL CANCER
ESTRADIOL
ESTRADIOL IN Pg/ml
16
12
8
4
14
2.0
NORMAL
CERVICAL CANCER

FIG - VIII a

Calibrator Levels: _____ Technician: _____

Analyte: SERUM ESTRADIOL Units: _____ Date Run: 18.2.'87

(MEAN) IN CERVICAL CANCER



Summary and Conclusion

V SUMMARY AND CONCLUSION

The summary of the results obtained from the study 'Biochemical and Hormone Profiles in Cervical Cancer' is discussed in this chapter.

Sixteen patients with cervical cancer from Kuppuswamy Naidu Memorial Hospital and five similar patients from Government Hospital, Coimbatore, participated for the present study. These women patients were of various stages of cervical cancer. According to the degree of severity of the disease, they were divided into different stages as stage I, II and III. Among the 21 patients who participated for the study, 5 of them were of stage I, 7 women of stage II and the remaining 9 patients of stage III of cervical cancer. Except one, all the others were of postmenopausal age.

The main aim of the present study was to find out whether there were any significant changes in the constituents of serum and plasma in cervical cancer patients. The selected biochemical parameters included ascorbic acid (in plasma), cholesterol, total creatinine, total protein, albumin, globulin, zinc, copper, iron and manganese (in serum). Besides these hormones like prolactin and estradiol were also analysed in the patients serum. The values obtained were

compared with those of normal healthy women who served as positive controls. Though inconclusive, the results obtained indicated that the above mentioned parameters may serve as diagnostic tools for cervical cancer.

The changes noticed in the selected parameters are summarised as follows.

Plasma ascorbic acid was found to be decreased significantly at 1 percent level in cervical cancer patients (0.4 mg/dl) when compared to the control group (1.4 mg/dl).

Cervical cancer < normal control group.

However, there was no significant change noticed between stages I, II and III of cervical cancer.

Plasma ascorbic acid level was diet dependant. Hence, low intake of ascorbic acid by cervical cancer patients led to a decrease in plasma level. But in the present study, there was no knowledge about the dietary intake of ascorbic acid by the patients.

In cervical cancer, the change in serum cholesterol levels depended on dietary fat consumption and also on the amount of steroid preparation drugs administered by the patients.

In the present study, there was a significant increase of cholesterol levels noticed in cervical cancer patients at 1 percent level compared to normal individuals (188 mg/dl). There was no significant difference between the various stages except that, a decrease at 5 percent level was noticed between stages II (258 mg/dl) and III (245 mg/dl).

As there may be more than one reason for the increase in serum cholesterol in cervical cancer, the exact reason for the increase in the present study seemed to be uncertain.

Increase in serum creatinine levels are generally associated with increased muscle breakdown and also renal failure.

It was seen that the serum total creatinine in cervical cancer increased significantly compared to normal subjects (1.0 mg/dl). The increase was significant at 5 percent level in stage II (1.6 mg/dl) and at 1 percent level in stages I (1.82 mg/dl) and III (1.75 ml/dl). However, there was no significant change between the various stages of cervical cancer.

The above results suggested that the increase in serum total creatinine may be probably due to the muscle breakdown and uraemia noticed in cervical cancer patients.

The results obtained in the present study showed a significant increase in serum total protein at 1 percent level in cervical cancer patients (8.4 g/dl) compared to those of normal women (7.02 g/dl). There was no significant difference between the various stages of cervical cancer.

Cervical cancer > normal control group.

While serum total protein increased, albumin level was found to be significantly decreased in cervical cancer compared to normal individuals (4.8 g/dl). The decrease was significant at 1 percent level. Between stages I (3.6 g/dl) and III (3.2 g/dl), a significant dedrease at5 percent level was also noticed.

Globulin level in serum showed a significant increase at 1 percent level in cervical cancer (5.0 g/dl) compared to the control group of women (2.82 g/dl). No significant difference was noticed between the different stages of cervical cancer.

Cervical cancer > normal control group.

Perhaps, the results of the above mentioned serum proteins may also serve as a diagnostic tool for the disease.

Serum zinc level was found to be low in cancer patients. The incidence of cancer was associated with dietary intake of zinc. On the other hand, this trace element also played a role in regulation of prolactin release.

In this study, the level of serum zinc decreased in cervical cancer patients compared to normals (98.5 µg/dl). The decrease was significant at 5 percent level and it was predominantly noticed in stage III (77.6 µg/dl).

The above results indicated that the decrease may be associated with debilitating disorder or renal failure as seen in cervical cancer patients.

The results obtained in the present study showed a significant increase in serum copper level in cervical cancer patients compared to normal women (114.7 µg/dl). The increase was significant at 5 percent level in stage II (158 µg/dl) and at 1 percent level in stage III (210 µg/dl). The increase thus paralleled the advancement of the disease as,

Stage I < Stage II < Stage III

Studies suggested that the serum copper levels increased in cancer. Perhaps the increase in serum level may also be diet dependant. But the dietary intake of copper in this study is not known.

Bleeding is one of the chief symptom noticed in patients with cervical cancer. Owing to this loss, the serum iron level decreases.

In the present study the serum iron level was found to be significantly decreased in the patients compared to normal control group. The decrease was significant at 1 percent level in stage III (49.3 µg/dl) compared to normal (97.5 µg/dl) and the other two stages. This clearly indicated that the decrease was associated with excessive bleeding particularly in the advanced stage of the disease.

Hence the serum iron level may be used as a marker in the diagnosis of cervical cancer.

Although there was no evidence for change in serum manganese level in cervical cancer patients, an attempt was made to analyse this trace element in serum.

The results obtained showed an increase in stage II (1.51 µg/dl) compared to normal (0.95 µg/dl). This

increase was significant at 5 percent level. There was no significant change between the different stages of cervical cancer.

The increase noted may be indicative of the post menopausal stage of the patients studied.

Prolactin was assayed in serum by 'Radio Immuno Assay' using the kit purchased from 'Diagnostic Products Corporation (DPC). Owing to the sophisticated and costly procedure, the hormone could not be analysed in the serum of all patients studied. The assay was conducted for 6 participants only and hence could not be sorted out into different stages of cervical cancer.

It was seen that the prolactin level increased in patients with cervical cancer (15.9 ± 19 ng/ml) compared to normal individuals (15 ng/ml).

Though inconclusive, this slight increase may be associated with drug intake or other disorders that interfere the normal hypothalamus inhibition of prolactin secretion. The exact reason for the increase is not known.

Serum estradiol was also assayed by RIA as that done for prolactin.

In the present study, serum estradiol was found to be lowered in cervical cancer patients (8 pg/ml) compared to normal (14 pg/ml).

There is no evidence for the changes in hormone level in cervical cancer. But an attempt has been made in this study to see whether cervical cancer was hormone dependent or not.

Suggestions for further research work

Further study in this field can prove whether cervical cancer results in any abnormalities or on the other hand a hormonal imbalance may precede the occurrence of cervical cancer. For this, progesterone levels may also help in diagnosis.

Besides these, quantitative intake of dietary ascorbic acid and trace elements like copper, zinc, iron and manganese may also help in concluding the results.

Thus, biochemical markers may aid in the diagnosis of cervical cancer and for this, further study is required.

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Appendices

APPENDIX I

ESTIMATION OF PLASMA ASCORBIC ACID

ROE AND KUETHER (Varley, 1976)

PRINCIPLE

The ascorbic acid is converted to dehydroascorbic acid by shaking with norit and this is then coupled with 2, 4 dinitrophenyl hydrazine in presence of thiourea as a mild reducing agent. Sulphuric acid then converts the dinitrophenyl hydrazine into a red compound which is assayed colorimetrically.

REAGENTS

1. TCA solution 60 g/litre
2. 2, 4 DINITRO PHENYL HYDRAZINE REAGENT

Dissolved 2 g of the solid in 100 ml sulphuric acid (1 part of sulphuric acid added to 3 parts water). Added 4 g thiourea and shook to dissolve. Filtered when necessary and kept in the refrigerator. To check whether enough thiourea was present, added the reagent drop by drop to 2 ml mercuric chloride. solution containing 10 g/litre. A copious precipitate of mercurous chloride formed after adding 2-5 drops.

3. ACID-WASHED NORIT

Placed 200 g Norit in a large flask, added a litre of hydrochloric acid (100 ml concentrated acid + 900 ml

water), heated to boiling and filtered with suction. Transferred the cake of norit to a beaker, added a litre of water, stirred thoroughly and filtered. Repeated until the washings showed a negative test for Fe^{3+} ions. Dried overnight at 110-120°C (not higher).

4. SULPHURIC ACID

Added 900 ml concentrated acid to 100 ml water.

5. STANDARD SOLUTION OF ASCORBIC ACID

Prepared a stock standard by dissolving 50 mg in 100 ml oxalic acid solution - 40 g/litre.

For the working standard diluted 2 ml of this to 100 ml with the acid used. This solution contained 10 mg/litre.

TECHNIQUE

To 6.0 ml TCA in a centrifuge tube added 2.0 ml plasma slowly with constant stirring to produce a fine suspension, stood 5 minutes, centrifuged then added 300 mg norit to the supernatant fluid. Shook vigorously and filtered. Placed 2.0 ml of the filtrate into each of 2 test tubes. Kept one for the blank and to the other, the test, added 0.5 ml, 2, 4 dinitrophenyl hydrazine reagent. Stoppered and placed in a water bath

at 37°C for exactly 3 hours. Removed and placed both test and blank in ice-cold water and added 2.5 ml sulphuric acid drop by drop, taking about half a minute to do so, so that there was no appreciable rise in temperature. Finally added 0.5 ml dinitrophenyl hydrazine to the blank. Mixed well the contents of both tubes while still in iced water. Removed and after 30 minutes read at 540 nm against the blank. As standard, treated 0.5 - 2.5 ml of the working standard in the same way as the test.

APPENDIX II

ESTIMATION OF SERUM CHOLESTEROL

ZAK'S METHOD (Varley, 1975)

PRINCIPLE:

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of the colour produced is directly proportional to the amount of cholesterol present and is read at 540 nm in a colorimeter.

REAGENTS:

1. STOCK FERRIC CHLORIDE REAGENT

840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.

2. FERRIC CHLORIDE PRECIPITATING REAGENT

10 ml of the stock ferric chloride was taken in a 100 ml standard flask and made upto the mark with glacial acetic acid.

3. FERRIC CHLORIDE DILUTING REAGENT:

8.5 ml of the stock ferric chloride was diluted to 100 ml with pure glacial acetic acid in a 100 ml standard flask.

4. STOCK CHOLESTEROL SOLUTION:

100 mg of pure dry cholesterol was taken in a clean dry 100ml standard flask and dissolved in 100ml of glacial acetic acid.

5. WORKING STANDARD SOLUTION:

10 ml of the stock standard wastaken in a 100 ml standard falsk containing 0.5 ml of the ferric chloride stock reagent and made upto the mark with pure galcial acetic acid. 1.0 ml of this solution contains 100/mg of cholesterol.

PROCEDURE:

0.5 to 2.5 ml of working standard cholesterol was pipetted out into a series of clean dry test tubes. The total volumn of each tube was upto 5.0 ml with ferric chloride diluting reagent.

To 0.1 ml of the serum added 4.9 ml of ferric chloride precipitating reagent and mixed well. Allowed to stand for a while and centrifuged. Transferred 2.5 ml of the clear supernatant into a dry test tube, added 2.5 ml of diluting reagent. Mixed well. The tubes were kept in cold water and to each tube added 4.0 ml of concentrated sulphuric acid drop by drop. The solutions were mixed well. The tubes were allowed to come to room temperature. A blank was also simultaneously prepared by taking 5.0 ml of the diluting reagent and 4.0 ml of concentrated sulphuric acid. After 30 minutes the colour developed was read at 560 nm.

APPENDIX III

ESTIMATION OF SERUM TOTAL CREATININE

ALKALINE PICRATE METHOD (Varley et al., 1980)

PRINCIPLE:

Creatine when treated with an alkaline picrate solution forms a red coloured complex. This is known as Jaffe's reaction. The intensity of the colour developed is compared in the colorimeter at 540 nm.

Blood contains both creatinine and creatine. Creatine is converted by boiling for one hour in the presence of picric acid. The total creatinine is found out from which the amount of creatine is calculated.

REAGENTS:

1. 0.04 M Picric Acid.
2. 0.075N Sodium Hydroxide.
3. Stock Standard

100 mg of creatinine was dissolved in N/10 hydrochloric acid and made upto 100ml with the same.

4. WORKING STANDARD

2.0 ml of the stock standard is diluted to 100 ml with water.

PROCEDURE:

To 3.0 ml of water, added 2.0 ml of serum, 1.0 ml of 10 percent sodium tungstate solution and 2.0 ml of 2/3N sulphuric acid. Kept for 10 minites and centrifuged. 3.0 ml of the supernatant was pipetted out into a test tube. Tubes containg 0.5 to 2.5 ml of the working standard solution were made upto 3.0mlwith water.
Along with this a blank was also prepared.
Added 1.0 ml of 0.04 N picric acid to all the tubes and 1.0 ml of 0.75 N sodium hydroxide and left for 20 minutes for the colour to develop. Shook well and the colour developed was compared with the standard against a reagent blank at 540 nm.

APPENDIX IV

ESTIMATION OF SERUM TOTAL PROTEIN, ALBUMIN AND GLOBULIN

BIURET METHOD (Varley et al., 1980)

PRINCIPLE:

The colorimetric method for protein estimation makes use of Biuret reaction. Substrates which contain -CONH$_2$ group joined directly on through a single carbon or nitrogen nitrogen atom gives a blue purple colour which is different for different proteins. The reaction takes its name from the complex formed that is Biuret.

REAGENTS:

1. STOCK BIURET REAGENT:

Dissolved 45g of Rochelle's salt in about 400 ml of 0.2N sodium hydroxide and 15g of copper sulphate pentahydrate, stirred continuously until the solution was complete. Added 5g of potassium iodide and made upto a litre with 0.2N sodium hydroxide.

2. DILUTE BIURET REAGENT:

Dilute 200 ml of stock reagent to a litre with 0.2 N sodium hydroxide which contained 5g of potassium iodide per litre.

3. STANDARD PROTEIN SOLUTION:

Weighed 400mg of albumin and dissolved in 0.9 percent sodium chloride solution so that 1.0 ml of this solution contains 4.0 mg of protein.

4. 0.9 PERCENT SALINE.

5. 22.5 PERCENT SODIUM SULPHATE SOLUTION:

PROCEDURE:

Into a serious of test tubes pipetted out 0.5 to 2.5 ml of standard protein solution. The volumn was then made upto 2.5 ml with water. Into another test tube added 0.2 ml of serum and diluted with 0.9 percent saline to 5.0 ml From this 2.5 ml of the solution was taken for the experiment. Now added 3.0 ml of diluted Biuret reagent to all the tubes Along with these blank was XX also taken. The colour developed was read at 500 nm colourimetrically after 30 minites.

A standard graph was drawn by platting concentration on X-axis and colorimeter readings on Y-axies. The amount of protein present was calculated.

PRECIPITATION OF GLOBUMIN:

Globumin was precipitated by mixing 0.2 ml of serum with 4.8 ml of 22.5 percent sodium sulfate solution.

Stoppered the test tubes and left in the incubator at $40^{0}C$. Filtered the solution next day using Whatmann No.42 filter paper. Took 2.5 ml of the filterate and carried out the experiment as for the test. The concentration of gram percentage of albumin present in globumin free filtrate was determined from the graph.

APPENDIX V

ESTIMATION OF SERUM ZINC, COPPER, IRON AND MANGANESE

PIPER'S METHOD (1969)

PRINCIPLE:

Serum on digestion with triple acid (nitric acid, sulphuric acid and perchloric acid in the ratio of 9:2:1) liberates into solution the trace elements.

PROCEDURE:

1.0 ml of the serum sample was taken in a micro kjeldahl digestion flask which was preciously washed with glass distilled waterand dried and to this was added 10 ml of triple acid. The mixture was shaken and digested on a sand bath with occasational shaking. The digestion was continued till no more brown fumes evolved. The digested mixure was transferred to a 25 ml standard flask, the washings being done with dobie distilled was water. This solution was used for analysing the trace elements, using the Atomic Absorption Spectrophotometer.

PROCEDURE FOR ROUTINE ANALYSIS:

1. Selected the lamps to be used and inserted them in the lamp quadrants.

2. Depressed the relevant LAMP- selected the button for the lamp being used and set the METER SELECT to the same lamp.

3. switched on the instrument. Set the lamp at the desired current and allowed to stabilish for 10-15 minites.

4. Set the indicator unit in the TRANSMISSION mode with the select switch in 'NORMAL'

5. Set the monochromator to the wavelength required with the relevant slit opening and using a gain setting to give approximately 80 percent T reading.

6. Select the desiredmode of operation on the indicator unit i.e. ABSORBANCE OR TRANSMISSION.

7. Selected the desired mode of 'Auto 100' mode and trimmed the 'SET 100' to read 0.0 Absorbance or 100 percent transmission.

8. Lighted the flame.

9. Nebulised the sample into the flame.

APPENDIX VI

ESTIMATION OF SERUM PROLACTIN

RADIO IMMUNO ASSAY @ DPC 1985)

PRINCIPLE:

The coat-a-count procedure is a solid phase Radio Immune Assay where in 125 I prolactin competes for a fixed time with prolactin in the patient sample for sites on prolactin- specific antibody. The antibody being immobilised to the wall of a polypropylene tube, decanting the supernatant suffices to terminate the competition and to isolate the antibody - bound fraction of the radio labelled prolation. Counting the tube in a gamma counter then yeilds a number which converts by way of a calibration curve to a measure of the prolactin present in the patient sample.

MATERIALS SUPPLIED:

1. PROLACTIN ANTIBODY COATED TUBES:

Polipropylene tubes coated with antibody to prolactin and packaged in zip lock bags stored refrigerated at 4 ° C.

2. BUFFERED (125 I) PROLACTIN:

One vial of lyophilised iodinated prolactin. Reconsituted each vial by addtion a measured 110 ml of distilled water. Let stand for 10 minutes, then mixed by

gentle inversion. Stored regrigerated at 4°C.

3. PROLACTIN CALIBRATORS:

Seven vials, labelled A through G, of lyophilized calibrators in processed human serum. Thirty minutes prior to use, reconstituted the zero calibrator A with 6.0 ml distilled water and each of the remaining calibrators B through G with 3.0 ml distilled water. Mixed by gentle inversion stored refrigerated at 4°C .

The calibrators were prepared in human serum which had been stripped of prolactin by affinity chromatography They represent 0, 5, 10, 20, 50, 100 and 200 ng of prolactin per ml (ng/ml). Intermediate calibration points can be obtained by mixing calibrators in suitable proportions.

PROCEDURE:

All components were kept at normal room temperature prior to use.

1. PLAIN TUBES:

Labeled four plain (uncoated) 12 x 75 mm polypropylene tubes T (total counts) and NSB (non specific binding) in duplicate.

2. COATED TUBES:

Labeled 14 prolactin antibody coated tubes A (maximum binding) and B through G in duplicated. Labeled additional antibody coated tubes, also in duplicate, for controls and patient samples.

CALIBRATOR	A (MB)	B	C	D	E	F	G
ng/ml.	0	5	10	20	50	100	200

3. Pipetted 200 /ul of the zero calibrator A into the NSB and A tubes and 200/ul of each remaining calibrator control and patient sample into the tubes prepared pipetted directly to the bottom.

4. Added 1.0 ml of buffered (125 I) prolactin to every tube. Vortexed briefly and gently.

5.(Set the T tubes aside for counting at step 6. They require no further processing)

5. Incubated for 18 hours at room temperature. (The tubes were covered with para film).

6. DECANTED THOROUGHLY:

Using a foam decanting rack, decanted the contents of all tubes (except the 7 tubes) and allowed them to drain for 2 or 3 minutes. Then struck the tubes sharply

on absorbent paper to shake off all residual droplets.

7. Counted for 1 minute in a gamma counter.

APPENDIX VII

ESTIMATION OF SERUM ESTRADIOL

RADIO IMMUNO ASSAY (DPC, 1985)

PRINCIPLE:

The coat - A count estradiol procedure is based on antibody coated tubes, 125 I labeled estradial competes with estradiol in the patient sample for antibody sites. After incubation, separation of bound from free is achived by simply decanting. The tube is then counted in a gamma counter, the counts being inversely related to the amount of estradiol present in the patient sample. The quantity of estradial in the sample is determined by comparing the counts to a standard curve.

MATERIAL SUPPLIED:

1. ESTRADIOL ♣ ANTIBODY COATED TUBES:

Polypropylene tubes coated with antibody to estradial were packaged in zip-lock bags, stored refrigerated at 4° C.

2. BUFFERED (125 I) ESTRADIOL:

One vial of dedinated estradiol in liquid form. Each vial contained 105 ml stored refrigerated at 2-8°C.

3. ESTRADIOL WALIBRATORS:

One set of 7 vials, labeled A through G of processed human serum. The calibrators A contained 5 ml. and the remaining calibrators B through G each contained 2 ml stored refrigirated at 2-8

The calibrators contained respectively 0, 20, 80, 150, 500, 1800 and 3600 picogrames of estimate estradiol per ml. (pg/ml) in processed human serum, equipvalently 0, 0.07, 0.18, 0.55, 1.84, 1.84, 6.61 and 13.20 n mol/lit.
Intermediate calibration points were obtained by mixing calibraters in suitable proportions.

PROCEDURE:

All components kept at normal room temperatures prior to use

1. PLAIN TUBES

Labeled 4 plain (uncoated tubes) 12 x 75 mm. polypropylene tubes T (total counts) and NSB (non specific Binding) in duplicates.

2. COATED TUBES:

Labeled 14 estradiol antibody coated tubes A (maximum binding) and B through G in duplicate. Labeled additional antibody coated tubesy also in dupliecate, for controls and patient samples.

CALIBRATORS	A (MB)	B	C	D	E	F	G
pg/ml	0	20	80	150	500	1800	3600

3. pipetted 100 µl of the zero calibrator A into the NSB and A tubes, and 100 µl of each remaining calibrator, control and patient sample into the tubes prepared. Pipetted directly to the bottom.

4. Added 1.0 ml of buffered (125 I) estradiol to every tube, vortexed.

5. Incubated for 3 hours at room temperature.

6. Decanted thoroughly.

7. Counted for 1 minute in a gamma counter.