MATERIAL AND METHODS
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The case material for the present study comprised of 22 healthy male and female volunteers of age 14-52 years.

The volunteers were selected from studies of previous years (1985-89) in the department of Medicine, M.L.B. Medical College, Jhansi and are as follows:

<table>
<thead>
<tr>
<th>Studies</th>
<th>Year</th>
<th>No. of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Arora RC and Gupta G</td>
<td>1985</td>
<td>3</td>
</tr>
<tr>
<td>2. Arora RC and Gupta VK</td>
<td>1986</td>
<td>8</td>
</tr>
<tr>
<td>3. Arora RC and Kumar K</td>
<td>1988</td>
<td>9</td>
</tr>
<tr>
<td>5. Arora RC and Mangal R</td>
<td>1989</td>
<td>5</td>
</tr>
</tbody>
</table>

Original studies of previous years mentioned above had a large list of volunteers from which, only limited number of subjects could be selected. It was largely because most of the previous volunteers had either left this institution or were not ready to reparticipate or were diseased group individuals or were not traceable.

All the selected volunteers are either medical students or employees of this college or children of college employees and every one of them is traceable within college premises. Moreover, few subjects among the total depicted in above table were common in studies
of previous years. Two female subjects had withdrawn from the test during high cholesterol fat breakfast (HCFB) and in two male subjects last (fifteenth day sample) sample could not be taken due to their resentments.

Informed consent was taken from each subject. A detailed history, thorough clinical examination and relevant investigations viz. blood sugar, blood, urea, TLC, DLC, Hb%, ESR, urine analysis and ECG were done in each case.

Use of oral contraceptives by female subjects and smoking, tobacco chewing and alcohol consumption in male volunteers was noted but not allowed during the whole period of study.

Detailed family history was enquired in every case regarding diabetes mellitus, ischaemic heart disease, hypercholesterolemia, hypertension, obesity, endocrinal disorders, or any other conditions which is likely to affect lipid metabolism.

Details regarding any substantial change in life styles or patterns viz. marriage, dietary changes, physical activity, use of any oral contraceptives in married female volunteers etc was noted.

Detailed dietary history was elicited to assess the amount of the fat consumed, daily and weekly in usual diet, amount of ghee and its type saturated or unsaturated oils and its type, milk and milk products,
eggs, food additives. In usual diet amount of fat and cholesterol was also noted. Most cases in this study were largely vegetarian and few who took nonvegetarian diet did so only once or twice in a fort night.

In those volunteers residing in hostel per head consumption of oil/ghee was calculated by giving consideration to total amount bought in mess and number of members eating in the same mess. No change in their usual diet was permitted just before and during the study period. Any recent medication (oral or parenteral) were also noted. P/S ratio of the usual diet was 0.4 : 0.6.

DESIGN OF TEST

The routine breakfast was substituted by high cholesterol fat breakfast (HCFB). Various combinations of HCFB were designed and given to volunteers approximating the diet protocol of original study series. However, the slight modifications were done so as to facilitate institution of diet in volunteers.

Those subjects who were common in many study series, were given, a single diet protocol of HCFB of the oldest study series.

HCFB was given under strict supervision.

The various HCFB used in this study were as below:
HCFB - I

It consisted of 3 eggs omlette in 20 gms of vanaspati ghee, four slices of bread approximately 20 gms each with 25 gms Amul butter, 250 ml sweetened whole fat buffalo milk and a cup of tea which was made optional.

This was given to eleven subjects of study series of 1985 and 1986 (Arora and Gupta G, 1985 and Arora and Gupta VK, 1987).

HCFB - II

It consisted of one boiled egg, four slices of bread approximately 20 gms each with 25 gms Amul butter, 250 ml sweetened whole fat buffalo milk and 500 mg of crystalline cholesterol dissolved in milk.

This was given to seven female subjects of study series of 1988 and 1989 (Arora and Kumar K and Arora and Pandey A, 1989).

HCFB - III

It consisted of two boiled eggs, 250 ml sweetened whole rat buffalo milk and 500 mg of crystalline cholesterol in milk. This was given to five subjects of study series of 1989 (Arora and Mangal R).

All the volunteers were asked to have their dinner at 8.0 PM on previous night and not to take anything except water thereafter. Next morning after
12-14 hours fasting the first fasting blood sample was
drawn in recumbent posture without producing venous
stasis (Koerselman et al, 1961). After this HCFB was
given and a second sample (post prandial) was drawn
after 1 hour and third sample (post prandial) after 3
hours of HCFB. On same day, during these three hours
subjects were not allowed any physical activity and
were largely confined to bed with only water in between
this period for drinking.

After this first day sampling the subjects
were routinely provided with HCFB daily morning for
seven consecutive days. On 8th day 12-14 hours after
fasting, fourth sample (fasting) was taken at 9:00 AM in
same way. From eighth day onwards HCFB was withdrawn
and volunteers were allowed their usual routine break-
fast and on fifteenth day fifth sample (fasting) was
taken at 8-9 AM after 12-14 hours fasting in recumbent
posture.

In whole period of study, except for HCFB,
the rest day's meal pattern was the same as was earlier
in their usual routine.

The serum of the collected samples was
separated within fours hours by centrifusing for
10 minutes and the supernatant of sample was put to
following tests:
1. Serum Total Cholesterol

This estimation was done by one step method utilizing the kit provided by Ortho Diagnostic System, Ethnor Division.

Procedure:

Three test tubes are taken and labelled as Test (T), Standard (S) and Blank (B) and then:

<table>
<thead>
<tr>
<th>Test (T)</th>
<th>STD (S)</th>
<th>Blank (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortho cholesterol reagent</td>
<td>4 ml</td>
<td>4 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>20 ul</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol standard (250 mg%)</td>
<td>-</td>
<td>20 ul</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mix contents of each test tube simultaneously for 10 seconds and immediately place them in a boiling water bath for exactly 45 seconds followed by cooling with running tap water or cold water for 5 minutes. Dry the exterior of tube mix their contents.

Measure optical density (OD) of each solution at 560 nm (range 560 to 600 nm). Set blank at calorimetric zero and calculation was done as

\[
\text{Cholesterol concentration of test samples (mg\%)} = \frac{\text{OD (T)}}{\text{OD (S)}} \times 250
\]

(Cholesterol mg/dl or mg\% / 38.7 = m mol/l).

(Range of normal expected values = 150 - 250 mg\%).
2. Serum Triglycerides (STG)

It was estimated by using GPO-PAP method for quantitative determination (Enzymatic method) of STG.

Procedure

The reagents in kit are:

4 vial of reagent I (Lyophilised enzymes)
1 vial reagent II (phenol solution)
1 vial of Triglyceride Standard (300 mg%)

Reconstitute reagent I in 2.5 ml of distilled water, while reagent II and triglyceride standard are supplied ready to use.

<table>
<thead>
<tr>
<th></th>
<th>Test (T)</th>
<th>Standard (S)</th>
<th>Blank (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Reagent II</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Mix well and add

Serum 20 ul

Triglyceride standard (300 mg/dl) 20 ul

Distilled water 20 ul

Mix and incubate in water bath at 37°C for 10 minutes.

Add distilled water & mix 2. ml 2 ml 2 ml

Take reading by measuring optical density (OD) of each solution at 500 nm (500-530 nm). Set blank at calorimetric zero. Calculation of STG is as:

\[
\text{STG in test sample (mg%)} = \frac{\text{OD} (T)}{\text{OD} (S)} \times 300
\]

(For conversion m mol/1 = mg/dl x 0.0114).
(Normal expected values = 30-150 mg%).

This test assay has validity for values up till 600 mg% only. If higher values expected then dilute serum suitably with distilled water and multiply value by dilution factor.

3. **Serum High Density Lipoproteins (HDL)**

It was estimated by kit provided by Ortho Diagnostic System, Ethnor Division.

**Procedure**

Two phases of test are involved:

**Phase I**: Lipogent™ agent (prepared solution for ready use) is used with serum to precipitate LDL and VLDL so that only HDL fraction remains.

**Phase II**: The supernatant of the above centrifuged solution is proceeded on with cholzyme-M kit for estimating HDL.

**Phase I**

Mix 0.5 ml of Lipogent™ with 0.5 ml of test serum. Keep at room temperature for 10 minutes and then centrifuge at 2000 rpm for 20 minutes to obtain a clear supernatant.

**Phase II**

Dilute one part of cholzyme M standard (200 mg%) with seven parts of distilled water = Cholzyme™ working reagent.
<table>
<thead>
<tr>
<th></th>
<th>Test (T)</th>
<th>Standard (S)</th>
<th>Blank (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholzyme™ working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Supernatant</td>
<td>100 ul</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Working standard</td>
<td>-</td>
<td>100 ul</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>100 ul</td>
</tr>
</tbody>
</table>

Mix and incubate in water bath at 37°C for 15 minutes.

Add distilled water 4 ml 4 ml 4 ml

Mix and read optical density (OD) by 515 nm (Range 500 - 530 nm) within 60 minutes.

Calculation is done as:

\[
\text{HDL concentration in test serum (mg\%)} = \frac{\text{OD (T)}}{\text{OD (S)}} \times 50
\]

(Conversion into SI unit \( \text{mmol/l} = \text{mg\%}/38.76 \))

(Range of normal expected values = 30 - 60 mg\%)

4. **Serum Very Low Density Lipoproteins (VLDL)**

   It was calculated by using formula given by Friedwald et al (1972):

   \[
   \text{VLDL (mg\%)} = \text{STG/5} \quad (\text{This formula is valid only upto STG value \( \leq 600 \text{ mg\%}.})
   \]

5. **Serum Low Density Lipoproteins (LDL)**

   It was also calculated by the formula given by Fredrickson DS (1972).

   \[
   \text{LDL (mg\%)} = \text{STC} - (\text{STG/5 + HDL})
   
   = \text{STC} - (\text{VLDL + HDL})
   \]
Charts were made for individual subjects and the pattern of change of lipid lipoprotein profile was noted. Remarks were specifically given for any marked change in any factors viz. marital status, occupation, physical activities, dietary habits, smoking, alcoholism, CAD in family history, fat consumption per day, history of any drug intake and finally conclusion was drawn regarding the change in lipid levels.

TABLE : Fat, cholesterol and fatty acids content in test meal.

<table>
<thead>
<tr>
<th>HCFB</th>
<th>Cholesterol (mg%)</th>
<th>Fat (gm%)</th>
<th>Saturated fat (gm%)</th>
<th>Polyunsaturated fat (gm%)</th>
<th>Monounsaturated fat (gm%)</th>
<th>P/S Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 egg (Hen)</td>
<td>250</td>
<td>6.0</td>
<td>2.2</td>
<td>0.85</td>
<td>3.00</td>
<td>0.4</td>
</tr>
<tr>
<td>Butter (25 gm)</td>
<td>70</td>
<td>21.5</td>
<td>12.9</td>
<td>0.64</td>
<td>7.95</td>
<td>0.05</td>
</tr>
<tr>
<td>250 ml milk (sweetened whole fat buffalo)</td>
<td>27</td>
<td>22.0</td>
<td>13.2</td>
<td>0.66</td>
<td>8.13</td>
<td>0.05</td>
</tr>
<tr>
<td>Crystalline cholesterol</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slices (4)</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: Fat, cholesterol and fatty acid contents were calculated on the basis of values given by Swaminathan MS: Essentials of Food Nutrients, (1974).