PRESENCE OF PROTEIN C DEFICIENCY IN PATHOGENESIS OF AVASCULAR NECROSIS (AVN) OF FEMORAL HEAD IN INDIAN POPULATION

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ABSTRACT

Despite multiple theories etiopathogenesis of AVN still remains an enigma and roughly 5%-40% of AVN cases remain classified as idiopathic in nature. The development of AVN in adults involves alteration in the balance of thrombophilia (enhanced tendency to form thrombi) and hypofibrinolysis (diminished ability to breakdown thrombi). There is dearth of such data in the Indian context. We devised this study to compare the incidence of protein C deficiency in patients with osteonecrosis of femoral head in comparison to occurrence in general population. We recruited 100 consecutive adult individuals of Indian origin in the study who were referred to our tertiary apex referral institute. All cases had clinically and radio graphically documented Avascular necrosis of the femoral head (AVN) of idiopathic nature. For our study group, suitably matched 50 control subjects for gender & age (1-year range) were recruited. The frozen samples were collectively run in batches for specific tests and further analysis. enzyme-linked immunosorbent assay (ELISA)- was utilized to measure antigen levels of protein C. We had 79 males & 21 Females in Cases &38 males & 12 females control group respectively. Mean age in Study group was 39.90 & control group was 41.3. AVN showed unilateral involvement in 78 cases & 22 cases showed bilateral involvement. We had 8 Hips in Ficat- Arlet Class 1 group, 34 in Stage 2, 32 in stage 3 & 48 hips in Stage 4 (Total 122 hips).9 cases out of 100 showed deficiency of Protein C (9%) as compared to no contr control group. AVN encompasses a complex interaction of both genetic & environmental factors and hence an individual person's susceptibility in developing AVN will depend on both environmental factors as well as genetic makeup. In the near future the stratification of this risk assessment would enable early diagnosis & also possibly a role of antithrombotics in disease prevention.

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INTRODUCTION

Osteonecrosis of the femoral head has been traditionally proposed to be the result of an ischemic insult sequentially causing avascular necrosis of subchondral femoral bone, femoral head collapse and ultimately degenerative changes at the hip joint. Patients belonging to age group of 30-50years are commonly indicted with Osteonecrosis. Majority of them require total hip replacement surgeries for permanent pain amelioration. Howsoever good the long-term results are yet the artificial joint tends to have a finite survival rate. This results in social & economic implications on the family& the society. Multiple risk factors such as alcoholism, steroids, hemoglobinopathies, vasculitis, dysbarism, autoimmune diseases and even pregnancy have been attributed to the development of Femoral head osteonecrosis (FHO). Despite multiple theories etiopathogenesis of AVN still remains an enigma and roughly 5%-40% of AVN cases remain classified as idiopathic in nature. It has been theorised that after thrombosis there occurs a sequential cascade of events

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progressing from obstruction of the venous drainage, consequent increase in venous pressure, inhibition of arterial perfusion, and ultimately into osseous necrosis. Analysis of published literature reveals that blood coagulation disorders including both thrombophilias & hypofibrinolysis play a role in AVN especially in Caucasian population. They analysed AVN cases especially the ones labelled as idiopathic and found significantly elevated incidence of these inherited thrombophilias. The most common thrombophilic disorders include G1691A mutation in the factor V gene (Factor V Leiden), G20210A mutation in the prothrombin gene, protein C deficiency, protein S deficiency, antithrombin deficiency, polymorphism in the plasminogen activator inhibitor-1 gene (4G/5G polymorphism), Antiphospholipid antibody, increased serum homocystine levels etc. The development of AVN in adults involves alteration in the balance of thrombophilia (enhanced tendency to form thrombi) and hypofibrinolysis (diminished ability to breakdown thrombi). The etiopathogenesis of Legg-Calvé-Perthes disease also involves the same thrombophilia /hypofibrinolysis mechanism. There is dearth of such data in the Indian context. We devised this study to compare the incidence of protein C deficiency in patients with osteonecrosis of femoral head in comparison to occurrence in general population.

MATERIALS AND METHODS

We recruited 100 consecutive adult individuals of Indian origin in the study who were referred to our tertiary apex referral institute from various parts of India. Informed written valid consent was taken from all subjects in study after obtaining institutional ethical committee clearance. Patients were followed up in the Department of Orthopaedics for the treatment of Avascular necrosis of Femur. All Cases had clinically and radiographically documented Avascular necrosis of the femoral head (AVN) of idiopathic nature. Each patient was diagnosed as having osteonecrosis of the head of the femur on the basis of a thorough history and physical examination, anteroposterior and frog-leg lateral radiographs of both hips, and magnetic resonance imaging as & when required. Ficat and Arlet classification was utilised for radiographic evaluation. MRI was performed to confirm the diagnosis of ONFH in patients with minimal x-ray changes. MRI criteria utilized were: 1) a focal bone signal anomaly with T1 and T2 hyposignal, 2) a peripheral medullar oedema with T1 hyposignal (rising up after a gadolinium infusion) and T2 hypersignal separated by delimitation border. This definition enabled detection in early stages as well as the elimination of differential diagnosis in doubtful cases. For our study group, suitably matched 50 control subjects for gender & age(1-year range) were recruited. The most vital criterion was to recruit controls non-affected by any renal or hepatic insufficiency or inflammatory syndrome, which could have in turn influenced thrombophilic factors. Majority of controls were recruited from patients who had been admitted for Degenerative spine disorders. Subjects were excluded from control groups if they were taking oestrogens, raloxifene, tamoxifen, corticosteroids, or anticoagulants.

Laboratory methods. Under all aseptic precautions, 10 ml venous blood of patient was collected in 3.2% buffered sodium citrate (one part citrate: nine parts blood). The sample was immediately transported and platelet-poor plasma was obtained with the help of centrifugation. The plasma was frozen & stored. The frozen samples were collectively run in batches for specific tests and further analysis. enzyme-linked immunosorbent assay (ELISA)- was utilized to measure antigen levels of protein C.

Principles of test are explained below

The determination was done using commercial kits. (Asserachrom Protein C – DiagnosticaStago, Asnieres, France)

Principle

The PC to be measured is captured by specific rabbit anti-human PC antibodies (Reagent 1) coated on the internal walls of a plastic microplate well. Next, rabbit anti-protein C antibodies coupled with peroxidase (Reagent 2) bind to the remaining free antigenic determinants of the bound PC. The bound enzyme peroxidase is revealed by its action on the TMB substrate (Reagent 3). After stopping the reaction with a strong acid, the intensity of the color is directly proportional to the concentration of PC initially present in the plasma sample.

Requirements

1. Citrated plasma samples of patients
2. Reagent 1 (R1): 16 well strip coated with specific rabbit anti-human PC F(ab)2 fragments
3. Reagent 2 (R2): specific rabbit anti-human PC antibodies coupled with peroxidase, freeze dried (to be reconstituted with 8ml of R4)
4. Reagent 3 (R3): ready to use tetramethylbenzidine (TMB< 1%) solution.
5. Reagent 4 (R4): ready to use phosphate buffer
6. Reagent 5 (R5): 20 fold conc. washing soln (to be diluted 1:20 with distilled water)
7. Reagent 6 (R6): freeze dried human plasma containing, after reconstitution with 0.5ml of distilled water, a known quantity of PC- Calibration
8. Reagent 7 (R7): freeze dried human plasma containing, after reconstitution with 0.5ml of distilled water, a known concentration of PC- Quality control
9. ELISA reader

Sample preparation

Patient’s plasma is diluted 1:51 in plastic test tubes with R4 (20 μl plasma + 1ml R4)

Calibration

The assay is calibrated with R6 diluted 1:51 in plastic test tube with R4 (20 μl R6 + 1ml R4). This dilution called starting solution contains the highest calibrator of value “t” given in assay insert. R4 is used to dilute the start solution as shown in

Table 1 Protein C ELISA Calibration
**Procedure**

**Table 2** Protein C ELISA protocol

<table>
<thead>
<tr>
<th>Pipette into each precoated well:</th>
<th>Test sample 200 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIGEN</strong></td>
<td>Cover the wells and incubate for 2 hours at room temperature (18-25°C).</td>
</tr>
<tr>
<td><strong>IMMOBILIZATION</strong></td>
<td>Wash all wells 5 times with R5, then immediately proceed:</td>
</tr>
<tr>
<td><strong>IMMOBILIZATION OF IMMUNOCONJUGATE</strong></td>
<td>R2 200 µl</td>
</tr>
<tr>
<td><strong>COLOR DEVELOPMENT</strong></td>
<td>Incubate each well at 18-25°C for exactly 5 minutes, then add acid:</td>
</tr>
<tr>
<td></td>
<td>1M H2SO4 50 µl</td>
</tr>
<tr>
<td></td>
<td>Swirl the plate to mix contents after H2SO4 added to all the wells.</td>
</tr>
<tr>
<td></td>
<td>Wait 15 minutes, and then measure the absorbance at 450 nm within one hour. Adjust the reader to zero on reagent blank.</td>
</tr>
</tbody>
</table>

Log-log paper is used to plot the calibrator values (%) on the abscissa (x-axis) and their corresponding absorbance values on the ordinate (y-axis). Using the calibrator curve, results (%) of the patients are read off directly.

**Normal Range:** 70-140%

**RESULTS**

Sample size was calculated for given study using statistical package “Epiinfo” (open/free source software). Study group of 100 cases & control group of 50 was selected using convenient sampling without personal bias. Our sample size was adequate to ascertain patient-control differences in our key measures of thrombophilia with alpha = 0.05 and beta = 0.2.

We had 79 males & 21 Females in Cases & 38 males & 12 females control group respectively. There was no significant difference between both groups with respect to age & gender. Mean age in Study group was 39.90 & control group was 41.3. AVN showed unilateral involvement in 78 cases & 22 cases showed bilateral involvement. We had 8 Hips in Ficat- Arlet Class 1 group, 34 in Stage 2, 32 in stage 3 & 48 hips in Stage 4 (Total 122 hips).

**Protein C deficiency**

9 cases out of 100 showed deficiency of Protein C (9%) as compared to no control showed deficiency of Protein C.

<table>
<thead>
<tr>
<th>Protein C Deficiency</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td><strong>Present</strong></td>
<td><strong>Absent</strong></td>
</tr>
<tr>
<td>Study Group with AVN</td>
<td>09 (9%)</td>
</tr>
<tr>
<td>Control group</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>09</td>
</tr>
</tbody>
</table>

The difference in proportion in Protein c deficiency in study & control group is statistically significant since p value is 0.028 (Mid-P exact test) which is <0.05. Odds ratio for developing AVN because of Protein C deficiency in study group is 9.791 times more than control group.

**DISCUSSION**

Avascular necrosis of Hip is one of the common reasons for middle age patients undergoing Total Hip replacement in Indian subcontinent in contrast to western world where AVN constitutes as Indication in only 10% cases undergoing THR. Various studies in literature mentions corticosteroid induced AVN in approx. 30 to 40% of cases, Alcohol use was found in 21 to 25% of cases, and the remainder of the cases are considered idiopathic. Our study specifically looked for thrombophilic profile of this idiopathic group, Protein C deficiency has autosomal dominant inheritance. Activated protein C (APC) inactivates coagulation factors Va and VIIIa, thus interfering with thrombin generation and factor X activation.

We found Protein C mutation in 9% of our study group with AVN femur in contrast we did not find Protein C deficiency in control group. Protein C was found to be significant determinant in Idiopathic AVN patients in Our study. Thus, there was 9.79 times higher risk of finding Protein C deficiency in Idiopathic AVN in comparison to healthy controls.

Garcia et al. also found Protein C deficiency in patients with Idiopathic AVN group than Secondary AVN group. In contrast Lee et al did not find the association of protein C to be of significance in patients with Idiopathic AVN in Korean population.

Variation in presence of protein C deficiency in the development of AVN in different population groups point towards a possible role of ethnicity on the genetic profile of risk for the development of AVN. AVN encompasses a complex interaction of both genetic & environmental factors and hence an individual person’s susceptibility in developing AVN will depend on both environmental factors as well as genetic makeup. In the near future the stratification of this risk assessment would enable early diagnosis & also possibly a role of antithrombotics in disease prevention.

**References and Bibliography**


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