



ROLE OF THROMPHOPHILIC MUTATIONS IN THE DEVELOPMENT OF AVN IN INDIAN POPULATION

Orthopaedic

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KEYWORDS

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^[24]

Patients belonging to age group of 30-50 years are commonly inflicted with Osteonecrosis. Majority of them require total hip replacement surgeries for permanent pain amelioration. However good the long term results are yet the artificial joint tends to have a finite survival rate. This portends pertinent social & economic implications on the family & the society¹.

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).^{2,3} Despite multiple theories etiopathogenesis of AVN still remains an enigma and around 5%-40% of AVN cases remain classified as idiopathic in nature.⁴

It has been theorised that after thrombosis there occurs a sequential cascade of events progressing from obstruction of the venous drainage, consequent increase in venous pressure, inhibition of arterial perfusion, and ultimately into osseous necrosis [9, 10, 28].

Analysis of published literature reveals that blood coagulation disorders including both thrombophilias & hypofibrinolysis play a role in AVN especially in Caucasian population^{5,6}. They analyzed 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 homocysteine levels etc.^{5,8}

The development of FHO in adults involves alteration in the balance of thrombophilia (enhanced tendency to form thrombi) and hypofibrinolysis (diminished ability to breakdown thrombi)^{5,6}. The etiopathogenesis of Legg-Calvé-Perthes disease also involves the same thrombophilia/hypofibrinolysis mechanism^{9,10}. There is dearth of such data in the Indian context. We devised this study to compare the incidence of thrombophilic risk factors in patients with osteonecrosis of femoral head in comparison to occurrence in general population.

MATERIALS & 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 classification¹¹ 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 estrogens, 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.

PCR analysis was utilised to study heritable thrombophilic gene mutation i.e. G1691A, also known as Factor V Leiden. Blood for polymerase chain reaction (PCR) analysis was collected in tubes containing the requisite amount of anticoagulant EDTA, and subsequent analysis was done with the help of the extracted DNA.

Antithrombin III levels were measured with the help of commercially available enzyme-linked immunosorbent assay (ELISA)-based assays. The cut-off levels of Antithrombin III to be considered abnormal for control subjects was kept at below the fifth percentile as per existing literature⁹.

Principles of tests are explained below-

AT activity by chromogenic assay

Principle

Commercial kits were utilized for AT activity assays. In the presence of heparin, AT exercises a strong immediate inhibitory action on thrombin. The test procedure consists of 2 steps. First, incubation of the test plasma is done with a recognized excess of thrombin in the

presence of heparin. Quantification of the residual thrombin is then done by its amidolytic action on the synthetic chromogenic substrate 61.50 (pNA release, 405 nm). Since the amount of thrombin that has been neutralized in the first reaction step is directly proportional to the AT level occurring in the test plasma, consequently we can imply that the amount of residual thrombin in the second reaction step (measured by pNA release) is inversely proportional to the actual AT level of the tested plasma.

Manual Method

Principle

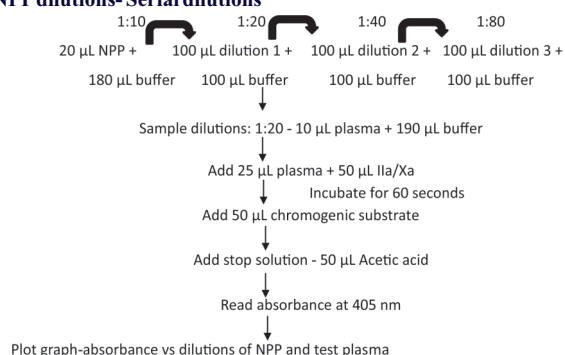
Assay is grounded on the capability of a plasma sample to inhibit a predetermined amount of thrombin (Factor IIa) / Factor Xa added in excess, in the presence of heparin. The residual enzyme is estimated by utilising chromogenic peptide substrate at 405nm.

Reagents

1. Reagent 1- Thrombin (IIa) / Factor Xa
2. Reagent 2- Chromogenic substrate
3. Reagent 3- Heparin.
4. Owrenkoller's buffer or Imidazole buffer
5. Acetic acid
6. DW
7. NPP

Method

NPP dilutions- Serial dilutions



Factor V Leiden (FVL) mutation (rs6025)

The terminology "factor V Leiden" denotes the specific G-to-A substitution at nucleotide 1691 in factor V gene (F5) that envisages a single amino acid replacement (R506Q) at one of the three APC cleavage sites in the factor Va molecule. Resultant factor V molecule is inappropriately inactivated by APC. Consequent to this dysregulation, the procoagulant properties of factor V are heightened in the coagulation cascade.

Principle

The overall size of the amplified product is 332 bp. MnlI restriction digestion of the wild type genotype leads to formation of three restriction fragments of 224 bp, 71 bp and 37 bp due to the presence of 2 MnlI restriction sites. The FVL point mutation (1691 G → A) destroys one of these two MnlI restriction sites, resulting in formation of two restriction fragments of 261 bp and 71 bp. Agarose gel electrophoresis helps in determination of genotype by analysis of the restriction digestion fragments. Bands of 71 bp and 37 bp are not visualised on 3% agarose gel or in a 10% PAGE.

PCR reaction Mix (30 µL)

PCR components	Volume (µL)
DNA (100-200 ng)	2
10X PCR buffer	3
Forward primer (10pM)	0.5
Reverse primer (10pM)	0.5
MgCl ₂ (25 mM)	1.5
dNTP (25 mM)	0.3
Taq DNA polymerase (5U/µL)	0.3
DW	21.9

Thermal Cycling Conditions

1.	Initial denaturation	94° C	5 minutes
2.	30 cycles		

a.	Denaturation	94° C	60 seconds
b.	Annealing	69 °C	120 seconds
c.	Extension	72°C	60 seconds
3.	Final extension	72°C	10 minutes
4.	Hold	4°C	∞

Resolution of digested fragments: Run the digested products on 3% agarose gel in 1X TAE or in a 10% PAGE in 1X TBE buffer.

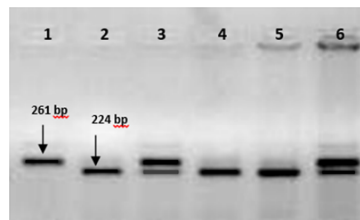


Figure 94: A representative gel showing different FVL genotypes

Factor V Leiden (FVL) mutation interpretation

Lane no (L-R)	Genotype	Fragment size (bp)
2, 4, 5	Normal	224, 71, 31
3, 6	Heterozygous	261, 224, 71, 31

Results –

Statistical package “Epiinfo” (open/ free source software) was utilized to calculate the sample size for the given study. Study group of 100 consecutive random 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 years & control group was 41.3 years. 78 cases had unilateral involvement & 22 cases showed bilateral involvement. The distribution according to Ficat- Arlet classification is given in Table no.1.

Ficat- Arlet Stage	Number
1	8
2	34
3	32
4	48

Antithrombin III deficiency -

ATIII deficiency was more prevalent in control group i.e. 22 % compared to 11% in study group.

	AT III Deficiency		Total
	Present	Absent	
Study Group with AVN	11 (11%)	89 (89%)	100
Control group	11 (22%)	39 (78%)	50
Total	22	128	150

AT III deficiency can not be considered to be a causative factor as deficiency of ATIII was found to be more common in control group as compared to study group.

Factor V Leiden Mutation-

Factor V mutation was present in 3 patients (3%) cases as compared to 1 patient (2%) in control group.

	FACTOR V LEIDEN Mutation		Total
	Present	Absent	
Study Group with AVN	03 (03%)	97 (97%)	100
Control group	01 (02%)	49 (98%)	50
Total	04	146	150

By virtue of the Mid-P exact test the p value was 0.393 which is > 0.05 and hence the difference in proportion in study & control group was not statistically significant.

DISCUSSION-

In the Indian population avascular necrosis of Hip is one of the most common indications for Total Hip replacement surgeries of middle age patients. This is in contrast to the Western world where AVN is an Indication in only 10% of cases undergoing THR¹². Analysis of literature pertaining to causes of AVN reveals that corticosteroid was

responsible in approx. 30 to 40% of cases, Alcohol in 21-25% of cases, and the remainder of the cases remain idiopathic¹³. We specifically studied the thrombophilic profile of this idiopathic group in our study.

In 1993 it was reported by Glueck et al in Twin brothers sustaining osteonecrosis of femur & found out on detailed analysis that they were homozygous for the hypofibrinolytic 4G/4G polymorphism of the plasminogen activator inhibitor-1 gene (PAI-1 gene). This mutation in turn caused the hypofibrinolysis state in blood¹⁴. Then in their landmark study they compared both thrombophilic mutations & hypofibrinolysis states in osteonecrosis⁵. They put forward the theory that these thrombophilic mutations cause increased interosseous venous pressure which leads to impaired arterial flow & hypoxia ultimately culminating into osteonecrosis.

As it is known that Thrombophilia is a state of hypercoagulability that is marked by inappropriate tendency to form blood clots. They found that inheritable thrombophilic disorders like elevated levels of factor VIII, Factor V Leiden mutation, and resistance to activated protein C, were risk factors for not only idiopathic but secondary osteonecrosis in femur as well.

Most of the published studies on heritable thrombophilic mutations have been carried out in certain geographical locations like specific parts of Europe, Morocco & America. As per our knowledge There is no study on thrombophilic mutations pertaining to AVN of femur in the Indian population. We sought to find out the answer to the question whether thrombophilia plays a pertinent role in the Indian ethnicity or not. Our study which consisted of 100 cases of idiopathic AVN with no known risk factors & 50 healthy controls highlights the role of thrombophilic mutations in AVN of femur in the Indian population. However in contrast to the study by Glueck, interosseous pressure was not measured by us. In contradistinction to most of the other published studies including the abovementioned study by Glueck, we had recruited healthy controls. Excluding of secondary risk factors of AVN was to eliminate confounding bias.

It is a known fact that the most significant inhibitors belonging to the coagulation cascade are antithrombin, protein C, and protein S¹⁵. The incidence of inherited deficiency of these is around 15%¹⁶. Heritable mutations commonly found in the general population that have been incriminated in causing thrombophilia consists of G1691A mutation in the factor V gene (factor V Leiden mutation), protein S deficiency, protein C deficiency, antithrombin deficiency & G20210A mutation in the prothrombin gene.

Antithrombin (AT) is a known serine proteinase inhibitor and alongwith its cofactor Heparin it is found to be a major determinant in the process of coagulation. As is evident from its name, Antithrombin deactivates thrombin, and alongwith that also inactivates factors IX, X and XI¹⁸. Contrary to expectation, we found out that Antithrombin III was not a significant mutation in the AVN group as compared to the control population.

Factor V Leiden normally activates prothrombin and helps in thrombin generation. Activated protein C (APC) inactivates Factor V by proteolysis. The G1691A point mutation in the factor V gene causes modification of the cleavage site at which APC degrades factor V. This in turn causes APC resistance & thrombophilia²⁰. Prevalence of the factor V mutation in healthy Caucasians has been reported to be around 4 to 5%²¹. In our study mutation in study group was found to be of 3 % in comparison to 2% in the normal control group population. This finding was not Statistically significant. On the other hand, Bjorkman et al.²² reported higher prevalence of factor V Leiden mutation in patients with idiopathic Osteonecrosis than that in the general Swedish population. However, a study done in Asian population i.e. Korea showed neither the presence of Factor V Leiden Mutation nor the Prothrombin gene mutation in patients with Idiopathic AVN which was similar to the findings of our study²³. Limitation of our study would be the limited size of control group.

Variation in thrombophilic mutations & their varied manifestations 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 would enable early diagnosis & also possibly a role of antithrombotics in disease prevention.

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