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Role of *NOS3* 894G>T Gene Polymorphism in Traumatic Cerebral Hemorrhagic Contusion Khartoum, Sudan, 2020-2021

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Abstract

The nitric oxide produced by the endothelial nitric synthase (NOS3) gene helps to maintain cerebral blood flow (CBF) after traumatic brain injuries (TBI). The aim is to determine the prognostic role of NOS3 894 G>T gene in traumatic hemorrhagic contusion and outcome. A cross-sectional study was conducted for 90 patients who attended the National Centre for Neurological Sciences, Khartoum, Sudan. Non-Sudanese patients, hemorrhagic contusions associated with other types of brain bleeding, and patients with chronic disease were excluded. An initial CT scan was used upon admission to detect brain edema, anatomical site, and the number of contusions. The Glasgow coma scale (GCS) was used upon admission to assess the trauma severity. The Glasgow outcomes scale (GOS) was used upon discharge to assess the outcome. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for NOS3 genotyping. The result shows that 93.3% of patients were male, while 32.2% of them were female. It further indicates that 58.9 % had a mild injury; 60% of the patients were presented with frontal lobe injury, 14.4% had multiple sites injury, and 22.2% had brain edema. The number of deaths was 8 (8.9%). The genotyping of NOS3 revealed that 94.4% of patients had homozygous GG and 5.6% heterozygous GT. G allele represented 97.2% of NOS3 alleles. NOS3 894 G>T genotypes were not significantly associated with patients, linguistic afflation' and outcome. The study concluded that NOS3 894G>T gene has no prognostic role in traumatic hemorrhagic contusion and outcome.

Keywords: *Nitric oxide synthase gene, Traumatic brain injury, Glasgow coma scale, Glasgow outcomes scale.*

Introduction

Traumatic brain injury (TBI) is a frequent cause of death and disability. It often leads to lifelong physical, cognitive, behavioural, and emotional impairments [1]. Damage of cerebral tissues following head trauma is determined by the primary injury plus secondary injury responses that are almost irreversible and worsen the primary injury [2]. A cerebral hemorrhagic contusion is a primary injury and often progresses during the first several hours after impact leading to either expanding or development of new non-contiguous hemorrhagic lesions [2]. Previous studies among Sudanese patients demonstrated that many factors were contributed to the progress of cerebral hemorrhagic contusion, including the genetic markers [3-5].

Nitric oxide (NO) plays a key role in the regulation of vascular homeostasis [6].

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Three main nitric oxide synthase (NOS) isoforms catalyzing the formation of NO have been well characterized. This family includes NOS1, NOS2 and NOS3, which are encoded by *NOS1*, *NOS2* and *NOS3* genes respectively [7, 8]. The inducible NOS (iNOS) produced by the *NOS2* gene lead to generating a high amount of NO to combat environmental insults. While neuronal NOS (nNOS) produced by *NOS1* gene and endothelial NOS (eNOS) produced by *NOS3* gene control a fluctuating low level of NO to perform normal physiological functions in neurons and vascular endothelial cells [9].

The Human Nitric oxide synthase (*NOS3*) gene mapped to the chromosomal locus 7q35-36 contains 26 exons that span 21 kb and encode a 135 KD, a protein containing 1203 amino acids [10]. *NOS3* gene has been determined for three allelic polymorphisms (- 786 C >T in the 5-flanking region, a 27-bp deletion(a)/insertion(b) in intron 4, and 894 G>T in exon 7) implicated in cardiovascular and cerebrovascular disorders [11]. The 894 G>T variant located in exon 7 is the most described one and has been suggested to be responsible for reduced NO synthesis [12, 13]. Trials propose that nitric oxide (NO) produced by *NOS3* gene participates in the regulation of cerebral blood flow (CBF) after TBI [14]. The observed reduction of NO in the injured brain results in lower CBF, hypo-perfusion, hypoxia, and an increase in intracranial pressure (ICP), therefore, associated with neurological deteriorations and poor clinical prognosis [14]. *NOS3* variants increase the ability of the injured brain to maintain an adequate CBF after TBI and may be associated with prognosis and outcome [14].

Accordingly, the timely determination of dependable prognostic factors for TBI patients is important for both neurosurgeon and clinical researchers. The potential to expect outcomes can be valuable in risk evaluation depending on the laboratory examinations and clinical features of the patients. A cerebral hemorrhagic contusion is a primary injury and can expand, leading to the delayed neurological deterioration [15].

The aim of this study is to investigate whether the *NOS3* 894 G>T gene polymorphism plays a role in the outcomes of traumatic cerebral hemorrhagic contusion. And to determine the genotyping distribution and allelic frequency for the *NOS3* gene among different Study participants.

Materials and Methods

Study Populations

A cross-sectional study was conducted at the National Center for Neurological Sciences (NCNS) from December 2015 to 2018. Ninety Sudanese patients who attended the emergency department at NCNS and were diagnosed with traumatic cerebral hemorrhagic contusion were included. Non-Sudanese patients, hemorrhagic contusions associated with other types of brain bleeding, and patients with chronic disease were excluded. An initial computed tomography scan (CT scan) was used upon admission to detect brain edema, anatomical site for contusion, and number of contusions. Glasgow coma scale (GCS) was used upon admission to assess trauma severity. The Glasgow outcomes scale (GOS) was used upon discharge to assess the outcome. Ethical approval was obtained from the Ethical Review Board of NCNS.

Data Collection and Genotyping

Blood samples were collected from all participants in an ethylene diamine tetraacetic acid (EDTA) tube. DNA was extracted from whole blood using QIAGEN® commercial DNA extraction kits (vacuum protocol)[16] attached was added ref No 16 DNA quantity and quality were

measured using Nano-drop spectrophotometer and gel electrophoresis. The following primers were used to amplify 894G>T region in *NOS3* gene (5'-CATGAGGCTC AGCCCC AGAAC-3) forward and (5'-AGTCAATCCCTTTGGTGCTCAC-3') reverse.

Polymerase chain reaction (PCR) was conducted into 20 µl reaction volume that contains 4 µl of 5X Firepol® master mix, 1 µl forward primer, 1 µl reveres primer, 1 µl DNA, and 13 µl distill water. PCR condition includes (Initial denaturation 95 C°/5 minutes, denaturation 95 C°/30 minutes, annealing 62 C°/30 minutes, extension 72 C°/30 minutes, and final extension 72 C°/10 minutes for 37 cycles). PCR product was loaded in 2 % agarose gel electrophoresis and visualized under UV light. PCR product 207bp was digested with MboI restriction enzyme obtained from *NEW ENGLAND BioLabs* ®, in 25 µl total reaction (1 µl of MboI restriction enzyme+ PCR product+ NEB buffer + H_2O). The mixture was incubated overnight at 37 C°, and then inactivated at 65C° for 20 minutes. The digested product was separated into 2% agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet light (UV light). Demographic and clinical data were collected from patients' records.

The Glasgow coma scale (GCS) was used upon admission to assess the trauma severity. The Glasgow outcomes scale (GOS) was used upon discharge to assess the outcome.

Statistical Analysis

Data analysis was carried out using Statistical Package for Social Sciences (SPSS) version 19. The data were described as frequency and percent, while quantitative data was described as (mean ± standard deviation). The chi-square test for independence was used to test the relations between qualitative nominal variables, while the Pearson correlation test was used to test quantitative variables. The independent sample T-test was used to test the difference between the two groups, and ANOVA was used in the case of three groups.

Results

In this study, 93.3% of the patients were male. The age of the most (32.2%) patients was 25 to 44 years. 67.8 % of the patients were affiliated to Afro-Asiatic tribes. The initial results of the computed tomography scan (CT scan) reflected that 60% of the patients were presented with frontal lobe injury,14.4% had multiple injured sites, and 22.2 % had brain edema. According to the GCS score, the majority (58.9 %) of the patients had a mild brain injury. According to GOS score, the number of deaths was 8 (8.9%). One patient died as a complication of severe pneumonia. The other seven died mostly of the primary insult.

NOS3 894 G>T gene showed two genotypes the homozygous GG represents 94.4% of the patients, and the heterozygous GT represents 5.6% of the patients. The most common allele of *NOS3* gene was G allele representing 97.2% of the alleles.

Table 1. Basic Characteristics of Patients on Admission

Variables	**Frequency**	**%**
Gender		
Male	84	93.3%
Female	6	6.7%
Age		
5-14	19	21.1 %

15-24	23	25.6 %
25-44	29	32.2 %
45-65	14	15.6 %
Over 65	5	5.6 %
Linguistic family		
Afro-asiatic	61	67.8 %
Nilo-Saharan	21	23.3 %
Niger-Congo	8	8.9 %
***GCS**		
Mild injury (13-15)	53	58.9 %
Moderate injury (8-12)	27	30.0 %
Sever injury (<8)	10	11.1 %
Anatomical site of trauma		
Frontal lobe	54	60.0 %
Temporal lobe	6	6.7 %
Parietal lobe	13	14.4 %
Biofrontal lobe	3	3.3 %
Occipital lobe	1	1.1 %
Others	13	14.4 %
Number of injured site/s		
Single	77	85.6 %
Multiple	13	14.4 %
Brain edema		
Yes	20	22.2 %
No	70	77.8 %
***GOS**		
Death	8	8.9 %
Neuro-vegetative state	0	0.0%
Sever disability	4	4.4%
Moderate disability	16	17.8%
Good recovery	62	68.9%

*GCS Glasgow coma scale *GOS Glasgow outcome scale

Table 2. Distribution of NOS3 894G>T Genotype and Allele Frequencies among Patients

***NOS3* 894G>T genotypes**	**Frequancy**	**%**
GG	85	94.4%
GT	5	5.6%
TT	0	0.0%
Total	90	100%
Allele frequencies of NOS3 894G>T gene		
G (Wild allele)	175	97.2%
T (Resistive allele)	5	3.8%
Total	180	100%

*NOS3 Endothelial cell nitric oxide-3 gene. G Guanine nucleotide. T Thymine nucleotide.

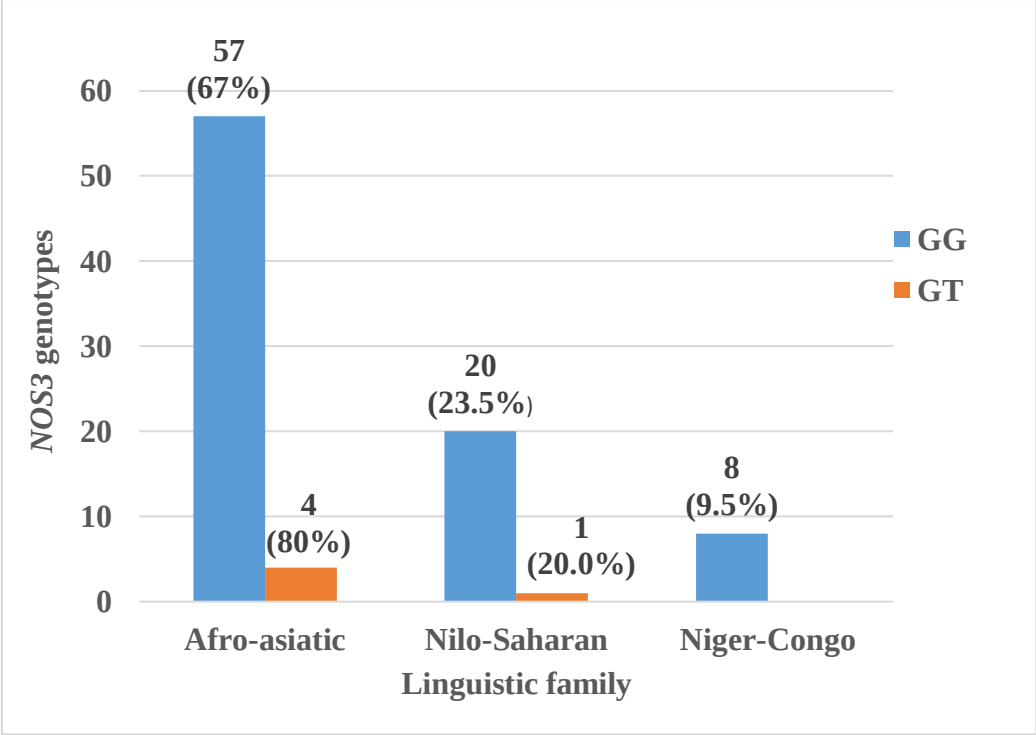

Figure 1. Association between NOS3 894G>T Genotypes Linguistic Family P value = 0.772

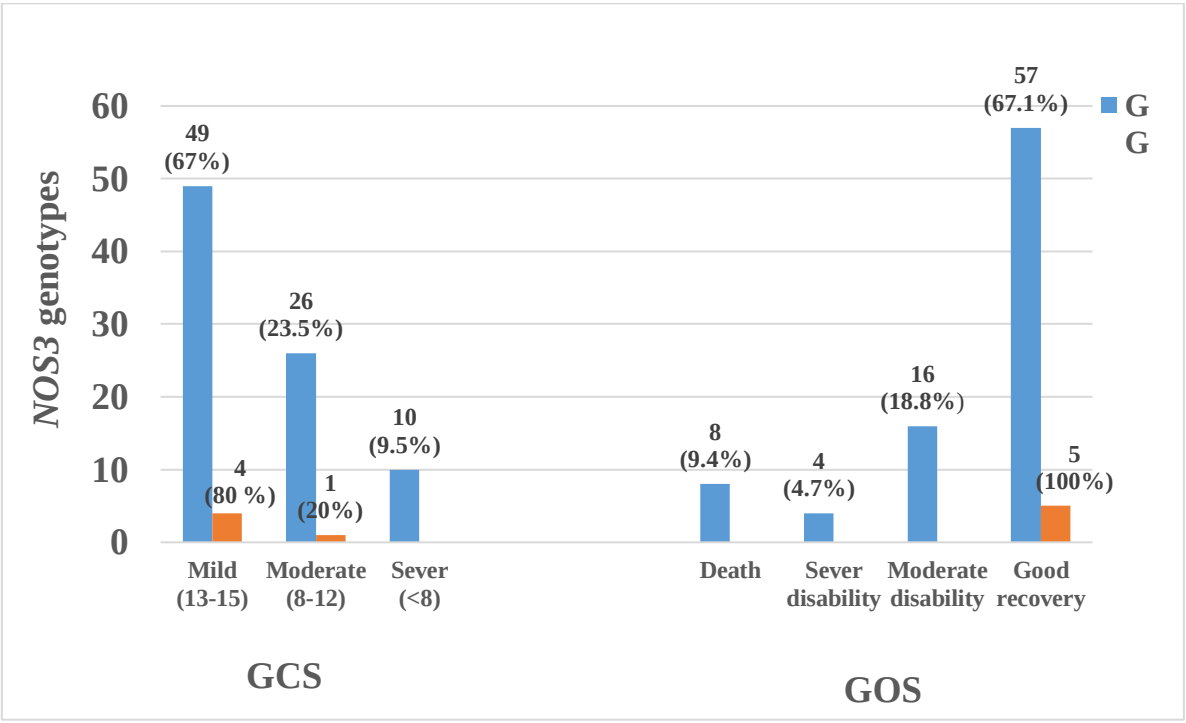

Figure 2. Association between NOS3 894G>T Genotypes with GCS and GOS

Discussion

[11]. studied three *NOS3* gene polymorphisms (-786 T>C, 894 G>T, and 27-bp VNTR) among severe TBI populations. They found a non-significant association between two Single nucleotide polymorphisms (SNPs) 894G>T and 27-bp VNTR with either mortality or CBF. Whereas -786 C allele was associated with low CBF and mortality [11]. Similarly, our findings confirm non-significant differences

between *NOS3* gene 894 G>T polymorphism with clinical features or death, but all the patients carrying T allele show good outcomes. The conflict of the results between the different studies can be attributed to the variability of the sample size or to the ethnic differences regarding the distribution of this pattern of polymorphism. Moreover, genetic association studies require biological evidence and functional significance that the risk variant is implicated in the pathogenesis of the disease [17, 18].

Some racial differences in the distribution of *NOS3* variants have been described that 894T allele was significantly more common in Caucasians than in African- Americans or Asians [19]. In the present study no significant distribution of *NOS3* variants among different Sudanese linguistic afflations while GT heterozygous allele was more common among Afro-asiatic participants. However, the sample size was too small to adequately assess this issue. No significant race/ethnicity differences in the distribution of the *NOS3* gene variants among American TBI victims [11]. Therefore, the genotypic and phenotypic studies should include larger sample sizes to determine how ethnic diversity may play a role in disease occurrence and disease outcome. However, further studies are needed since the predicting outcome following TBI is challenging and cannot be made based solely on clinical presentation, and radiological findings since patients with comparable injuries may have variable outcomes [20].

Conclusions

The study concluded that *NOS3* 894G>T gene polymorphism has no prognostic role in traumatic cerebral hemorrhagic contusion, clinical features, and outcome.

Recommendation

A national program targeting the young population for the prevention and management of traumatic injury should be in place.

A large-scale study is needed to explain some of the variability in outcomes that occur following traumatic cerebral hemorrhage.

Conflict of Interest

We declared no conflict of interest in this article.

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