Role of endothelial nitric oxide synthases system on acute appendicitis

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ABSTRACT

BACKGROUND: Obstruction and inflammation of the appendix lumen is the leading physiopathological process during acute appendicitis (AA). Although the relationship between inflammation and endothelial nitric oxide synthases (eNOS) has been well described, no recent data describing the relationship between inflammation during AA and polymorphism of the eNOS gene has been reported. Given the limited data available, we believed that defining the relationship between AA and eNOS would be a beneficial contribution.

METHODS: A total of 201 patients admitted to the emergency department with AA and 201 healthy volunteers selected from among the relatives of patients were included. Polymorphism of the eNOS was assessed.

RESULTS: Intron 4a/4a was positive in 119 participants, genotype G894T GT was positive in 71 patients with AA, and 786-1 was positive in 71 patients with AA. These results suggest that no statistically significant correlation exists between genotypes of AA patients and control subjects regarding 4a/b, G894-GT, and 786-1 eNOS polymorphisms.

CONCLUSION: Though the present results suggest that no statistically significant correlation exists between AA and eNOS gene polymorphism, to claim otherwise is also impractical. We believe that the present results will lay the groundwork for future, larger studies.

Keywords: Acute appendicitis; genetic polymorphism; nitric-oxide-synthases.

INTRODUCTION

Acute appendicitis (AA), a leading cause of emergency department admittance, has been the subject of many studies. The primary pathophysiological process behind AA has been accepted as the obstruction of the appendix lumen and consequent inflammation.[1-3] However the relationship between inflammation and obstruction has yet to be clearly defined—which is the cause and which the effect remains vague. Likewise, obstruction of the appendix lumen by an ingested fruit pit may cause AA, though not all who ingest a pit will develop AA. Therefore, there is need for further study of the inflammatory process and development of AA.

Nitric oxide (NO), a well-studied signal molecule, is located within the smooth muscle cells and plays a role not only in several gastrointestinal functions, but also in defense system and immunologic reactions. During inflammatory processes, NO was found to be an important factor in vasodilatation, changes in vascular permeability, extravasation, and leukocyte migration and activation.[4,5] Genetic polymorphism of endothelial nitric oxide synthases (eNOS), an isoform of nitric oxide synthases (NOS), which is an enzyme in NO synthesis, is correlated to basal NO levels.[6] It has been suggested that NO levels rise in cases of AA.[7-9]
While eNOS has been described as closely related to inflammation,[10] few studies have focused on the relationship between inflammation in AA and genetic polymorphism of eNOS. The focus of the present study was eNOS gene polymorphism in patients with AA.

**MATERIALS AND METHODS**

The present study was conducted via collaboration of the departments of emergency medicine and medical biology at Erciyes University, a tertiary hospital with a catchment area of 15 million, and an emergency department admittance of 110,000. It was approved by the local ethics committee (on August 15th, 2014; no: 490). Blood samples were obtained from AA patients who presented to the emergency department and were genetically analyzed in the Genome and Stem Cell Center (GENKOK), located in the same university. Procedures were performed by an experienced emergency department physician who was not involved in patient treatment.

**Patient Selection and Sampling**

Included were those who presented to the emergency department with symptoms of stomachache accompanied by nausea, vomiting, and loss of appetite (known as “first 6-hour signs”) and who were diagnosed with AA following routine workup. Patients who died shortly after surgery and who were not pathologically diagnosed with AA were excluded. Routine blood workup included complete blood count (CBC), neutrophil-leukocyte ratio, glucose, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), sodium, potassium, chloride, lactate dehydrogenase (LDH) levels, and direct and indirect bilirubin. There was no age limit as inclusion criteria. Routine radiological workup consisted of ultrasonography, computed tomography, or both. This study did not affect the diagnosis or treatment of AA. Complaints, signs and symptoms, and CBC were computed according to the Alvarado scoring system.[12] This system, with a maximum score of 10, has 8 parameters, 3 of which are related to symptoms, 4 of which are related to clinical signs, and 3 of which are related to CBC.[13] Higher scores more strongly indicate AA, with a sensitivity of 80%.[14] A score of below 5 indicates decreased likelihood of AA, a score of 5 or 6 indicates a weak likelihood of AA, a score of 7 or 8 indicates possible AA, and a score of 9 or 10 indicates a strong probability of AA.

The control group consisted of volunteers, selected among the relatives of AA patients. Similar blood sampling was performed in the control group. Following oral and written consent, 2 mL of blood was drawn into tubes containing ethylenediaminetetraacetic acid as anticoagulant. Samples were kept at +4°C until genetic analysis.

**Genetic Analysis**

All genetic studies were conducted in the GENKOK laboratory of Erciyes University. eNOS gene polymorphisms were analyzed following genomic DNA extraction from peripheral blood leukocyte samples of all participants, using a High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics International AG, Rotkreuz, Switzerland). For the detection of the eNOS-786 TC polymorphism, PCR-restriction-fragment-length polymorphism analysis was used. PCR reaction was carried out using 10 pmol of each primer: 5’-AGGCCCTATGGTAGTGCCTT-3’ (forward) and 5’-TCTCTTAGTGCTGTGTCAC-3’ (reverse). For each PCR, 10 ng DNA was amplified in a final volume of 50 μL at an annealing temperature of 60°C. After PCR detection, Mscale (Thermo Fisher Scientific, Inc., Waltham, MA, USA) restriction endonuclease digestion was performed for 16 h at 37°C. Final PCR products were analyzed by electrophoresis on 3% agarose gel. The eNOS 4a/4b polymorphism was detected only by PCR amplification. PCR reaction was carried out using the primers 5’-AGGCCCTATGGTAGTGCCTT’- (forward) and 5’-TCTCTTAGTGCTGTGTCAC-3’ (reverse). This procedure was based on DNA amplification in a final volume of 50 μL, followed by heating at 56°C. PCR products were analyzed by electrophoresis on 2% agarose gel.[15] Ten μg of genomic DNA of each participant was amplified by PCR for eNOS G894T polymorphism. The 50-μL reaction mixture contained forward 5’-CATGAGGCTCAGCCCCAGAAC-3’ and reverse 5’-AGGCCCTATGGTAGTGCCTT-3’ primers, amplifying at 60°C. PCR products were then subjected to overnight incubation at 37°C with 1U of Mbol restriction endonuclease (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Digestion products were separated using 2.5% agarose gel electrophoresis and stained in ethidium bromide (Table 1).[16]

**Statistical Analysis**

Data were evaluated with SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). Descriptive statistics and inferential statistics were used for evaluation. Shapiro-Wilk test was conducted to assess normality, and Bonferroni test was used in multivariate analysis. Strength of relation between variables was measured with Pearson correlation analysis. Discrepancy of age between patient and control groups was compared with Student’s t-test, and categorical variables were compared with chi-square test. A value of p<0.05 was accepted as statistically significant.

**Table 1.** Polymerase chain reaction program and restriction enzymes for genotyping eNOS gene polymorphisms

<table>
<thead>
<tr>
<th>eNOS gene polymorphism</th>
<th>Restriction enzyme</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introns 4 (4a/4b) (rs61722009)</td>
<td>–</td>
<td>56</td>
</tr>
<tr>
<td>894G&gt;T (rs1799983)</td>
<td>Mbol</td>
<td>60</td>
</tr>
<tr>
<td>-786 T&gt;C (rs2070744)</td>
<td>Mscale</td>
<td>60</td>
</tr>
</tbody>
</table>
RESULTS

Clinical and laboratory findings for study and control groups

The patient group consisted of 88 females and 113 males between ages of 3 and 80 years (mean: 29.6±15.8). The control group consisted of 92 females and 109 males with a mean age of 29.7±16.0 years. There was no age discrepancy between groups (p>0.05). In addition, correlation of age and gender parameters with eNOS intron 4 genotypes was not statistically significant (p>0.05) (Table 2).

Primary complaints in the patient group were: stomachache in 147 patients (73%), nausea in 14 (7%), vomiting in 14 (7%), loss of appetite in 8 (4%), and all 3 signs in 18 patients (9%) (Table 3).

Regarding vital signs, body temperature was between 36oC and 38.3°C (mean 36.7±0.58°C). Systolic and diastolic blood pressures were 90–180 mmHg (mean: 121.1±13.5 mmHg) and 41–113 mmHg (mean: 73.2±11.4 mmHg), respectively. Respiration rate was 18–28 bpm (mean: 20.1±1.4 bpm). Cardiac pulse rate was measured as 68–103 beats per minute (mean: 87.4±21.5 beats per minute) (Table 4).

CBC provided the following data: white blood cell count had a mean of 14.1±3.8 mg/dL (min-max: 7.8–36.2 mg/dL); thrombocyte count had a mean of 240.0±89.3/mm³ (min-max: 104–594/mm³); hemoglobin count had a mean of 13.6±1.9 mg/dL (min-max: 8.2–17.1 mg/dL); and neutrophil-lymphocyte ratio had a mean of 3.7±1.1 (min-max: 1.9–6.9). Blood glucose levels were 56–147 mg/dL (mean: 97±11.9 mg/dL), BUN was 4–50 mg/dL (mean: 13.5±6.3 mg/dL), creatinine was 0.09–1.55 mg/dL (mean: 0.6±0.2 mg/dL), AST was 6–55 U/L (mean: 21.3±9.9 U/L), ALT was 3–89 U/L (mean: 16.5±11.6 U/L), sodium was 131–144 mmol/L (mean: 139.2±19 mmol/L), potassium was 3–5.7 mmol/L (mean: 4.2±0.4 mmol/L), total bilirubin was 0.1–5.3 mg/dL (mean: 0.7±0.5 mg/dL), and LDH 142 was 652 IU/L (mean: 254.0±103.9 IU/L) (Table 5).

A total of 116 patients were evaluated with ultrasonography, 48 patients with computed tomography, and 37 patients with both methods (Table 6). Using the Alvarado system, 12 patients (6%) had a score of 5 or 6, 128 patients (64%) had a score of 7 or 8, and 61 patients (30%) had a score of 9 or 10.

Genetic Analysis Data

Endothelial NOS genotype and allele distribution for each group were determined. From among the 3 genotypes for intron 4, 12 participants had genotype 4a/4a, 119 participants had genotype 4b/4a, and 271 participants had genotype 4b/4b. Of the participants with genotype 4a/4a, 7 (58%) were from the patient group, and 5 (42%) were from the control group. Of the participants with genotype 4b/4a, 60 (50%) were from the patient group, and 59 (50%) were from the control group. Of the participants with genotype 4b/4b, 134 (49%) were from the patient group, and 137 (51%) were from the control group. Distribution of genotypes between patient and control groups were statistically insignificant (p=0.852) (Table 7).

The GG genotype of eNOS-894 polymorphism was found in 112 patients (56%) and 119 healthy volunteers (60%), the TT genotype was found in 18 patients (9%) and 16 healthy volunteers (8%), and the GT genotype was found in 71 patients (36%) and 66 healthy volunteers (33%). Correlation between patient and control groups regarding eNOS-894 polymorphism was not statistically significant (p=0.774) (Table 7).

Table 2. Gender parameters

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study group n (%)</th>
<th>Control group n (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>113 (51)</td>
<td>109 (49)</td>
<td>222</td>
</tr>
<tr>
<td>Female</td>
<td>88 (49)</td>
<td>92 (51)</td>
<td>180</td>
</tr>
<tr>
<td>Total</td>
<td>201</td>
<td>201</td>
<td>402</td>
</tr>
</tbody>
</table>

χ²= 0.161  p=0.688

Table 3. Main complaint at admittance

<table>
<thead>
<tr>
<th>Complaint at admittance</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomachache</td>
<td>147</td>
<td>73</td>
</tr>
<tr>
<td>Nausea</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Vomiting</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Stomachache, nausea, vomiting</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>201</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4. Vital signs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean±SD</th>
<th>Minimum-Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Temperature (°C)</td>
<td>36.7±0.58</td>
<td>36–38.3</td>
</tr>
<tr>
<td>Systolic/diastolic (mmHg)</td>
<td>121.1±13.5/73.2±11.4</td>
<td>90–180/41–113</td>
</tr>
<tr>
<td>Respiratory rate/min</td>
<td>20.1±1.4</td>
<td>18–28</td>
</tr>
<tr>
<td>Cardiac pulse rate/min</td>
<td>87.4±21.5</td>
<td>68–103</td>
</tr>
</tbody>
</table>

SD: Standard deviation.
The 786-1 genotype of eNOS-786 polymorphism was positive in 71 patients (36%) and 80 healthy volunteers (40%), the 786-2 genotype was positive in 6 patients (3%) and 8 healthy volunteers (4%), and the 786-3 genotype was positive in 124 patients (62%) and 113 healthy volunteers (57%). These results showed no statistically significant relationship between the patient and volunteer groups (p=0.514) (Table 7).

DISCUSSION

The present focus was on the probable involvement of eNOS in the pathophysiology of AA. Though the role of eNOS in inflammation processes has been well defined, no statistically significant findings were presently reached, a conclusion that is discussed below. Still, the present results also point to the difficulty in denying the existence of this relationship. Definitive conclusions require further study, utilizing larger patient and control groups. We believe that our findings have potential importance, but that the limited number of included cases prohibits the formation of precise conclusions. We consider the present to be an entry study in the description of a possible relationship between eNOS and AA inflammation.

The 786-1 genotype of eNOS-786 polymorphism was positive in 71 patients (36%) and 80 healthy volunteers (40%), the 786-2 genotype was positive in 6 patients (3%) and 8 healthy volunteers (4%), and the 786-3 genotype was positive in 124 patients (62%) and 113 healthy volunteers (57%). These results showed no statistically significant relationship between the patient and volunteer groups (p=0.514) (Table 7).

The most important physiopathological process in the etiology of AA is thought to be the obstruction of the appendix lumen due to fecaloma or other factors that cause inflammation.[17–19] On the other hand, as not all cases with such factors advance AA, and other components may have a physiopathological role in AA.

NO, which has been studied in this context since the 1980s, is a free radical molecule that is chemically unstable in nature, but essential in local vascular stability. Its half-life is very short, 5–10 seconds, and it has several anti-inflammatory, anti-oxidant, and anti-fibrotic functions in the relaxation of smooth muscle and aggregation of leukocytes and thrombocytes.[20–22]

The present study was based on the hypothesis that AA inflammation may be caused by a defect in the NO mechanism, which functions normally in anti-inflammatory processes, challenging the finding stated elsewhere that NO levels rise in cases of AA. Our opposition was due to the short half-time of NO, its unstable nature, and other factors yet unknown. It is known that NO, an inhibitor in the coordination of tonic and motility functions throughout the gastrointestinal tract, from the esophagus to the anal sphincter, also has secretory influence in physiopathological conditions.[23] Given the complicated nature of these processes and physiopathological circumstances, the relationship between NO and AA seems yet to be clearly understood. Our results suggest that the working mechanism of NOS, which is involved in the synthesis of NO, is unable to account for the entire mechanism in cases of AA. We believe that this is related to the 3 different isoen-
zymes of NOS, which will hopefully be the subject of future studies. The eNOS gene is located on the terminal part of 7q32-q, and its polymorphic structure has been described in several pathologies, including coronary heart disease and hypertension. Similarly, it is thought that AA may have a genetic background. In more detail, a polymorphism in the gene that codes the eNOS enzyme may affect gene expression, which may cause eNOS activity, and therefore inflammatory response, leading to AA.

Patients presently evaluated were carefully chosen from among those admitted to the emergency department with AA. Clinical and laboratory features of patients were presented in the results section, but are not discussed here, as they are irrelevant to the present genetic focus.

The patient and control groups were evaluated regarding allele and genotype distribution. Intron 4 positivity was investigated in 3 genotypes, and the most common genotype with intron 4 positivity was identified as homozygote 4b/4b. However, this result was not statistically significant, compared to frequency in other groups. Regarding the heterozygote 4b/4a genotype, there was statistically significant difference between groups. However, positivity was identified in around 60 individuals, from among the control and patient groups. No significant polymorphism regarding the intron 4 genotype in AA patients was identified in this initial study. Future studies will include other types of polymorphism.

In AA patients, the eNOS-894 GG genotype was detected in 112 patients, the eNOS-894 GT in 71, and the eNOS-894 TT in 18. However, these results had no statistical significance. The eNOS-786-TT, eNOS-786-TC and eNOS-786-CC genotypes were found in 71, 6, and 124 patients, respectively. As the present findings had no statistical significance, we believe that there is no relationship between AA and eNOS gene polymorphism.

Conclusion

We conclude that eNOS gene polymorphism is not related to AA.

Acknowledgement

This study was supported by a project (TTU-2014-5595) from the Scientific Research Projects Department of Erciyes University, Kayseri, Turkey.

Conflict of interest: None declared.

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Akut apandisitte endotelial nitrik oksit sentaz sisteminin rolü

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AMAC: Akut apandisit etiyolojisinde önemli fizyopatolojik sürecin apandiks lümeninin bir nedenle obstrüksiyonu ve enfamasyonu olduğu bilinmesine rağmen, bu enfamasyon ile endotelial nitrik oksit sentaz (eNOS) gen polimorfizmi arasında yapılan bir araştırma bulunmamaktadır. Oysaki enfamasyon ile endotelial nitrik oksit sentaz ilişkisi birçok çalışmada gösterilmiştir. Literatürdaki eksik yerini hala koruyan akut apandisit ve endotelial nitrik oksit sentaz gen polimorfizmi muhtemel ilişkisi hipotezi araştırılmasa da değer bulunmuştur.


BULGULAR: İntron 4b/4a’nın 119 bireyde pozitif olduğu görüldü. Genotiplerden G894T-GT’nin 71 ve nitrik oksit sentaz 786-1’in 71 akut apandisitli hastalarında çalışma grubu oluştururken, yaklaşık 402 bireyle çalışılmasına da kontrol grubu belirlendi. Çalışmada eNOS gen polimorfizmine ait genotipleri analiz edildi.

TARTIŞMA: Akut apandisit ve eNOS gen polimorfizmi muhtemel ilişkisi olarak hipotezi kurulan bu çalışmada edilen sonuçlar eNOS ile akut apandisit arasında istatistiksel bir ilişki kurulmamıştır. Ancak, böyle bir ilişkinin olmadığı bu çalışma ile iddia etmek mümkün gözükmemektedir. Metodolojik açıdan elde edilen veriler önem taşımaktadır ve yapılacak daha geniş çalışmalar ile bu konu hakkında daha detaylı bilgiler elde edilebilecektir.

Anahtar sözcükler: Akut apandisit; genetik polimorfizm; nitrik- oksit-sentaz.