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Original Research

Detection of cyclooxygenase -2 gene polymorphism among Sudanese women with recurrent spontaneous abortion in Khartoum state, Sudan in 2021

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ABSTRACT

Background: Recurrent spontaneous abortion is a health issue that occurs in about 1 — 5% of all women of reproductive age. Experimental evidence suggests that COX-2 plays an important part in blastocyst implantation but rarely is the role of COX-2 recognized in recurrent spontaneous abortions. **Methods:** This is a descriptive analytical case-control hospital study that was carried out at the Research Laboratory of the National Center for Neuroscience in Khartoum, Sudan from April to September 2021. The study intended to detect cyclooxygenase-2 gene polymorphism among Sudanese women with RSA. Women with more than three recurrent miscarriages were included in this study as the sample cases and women with no history of abortion at child-bearing age were included as the controls. From each of the subjects, three ml of venous blood was gathered in sterile containers with Ethylene Diamine Tetra-acetic Acid (EDTA). The DNA was isolated using the standard phenol chloroform extraction method. The COX-2 gene was amplified using conventional PCR. The products of the PCR were sent to the Macro Gene Laboratory for sequencing. **Results:** In the current research, 230 bp of C0X-2 gene was disclosed using gel electrophoresis after PCR. The results for the detected gene from all subjects were analyzed. The X2-test showed that there were significant differences in the gene expression and distribution between the case and control groups (p value 0.001). The sequence results analysis showed that there was a change in C>A at Location 1:633149 (Rs1481253765) and a change in G> T cDNA position 427 at Location 1:633183 (Rs1232472838). **Conclusion**: This study showed that there was a consequential difference in the COX-2 allele between the case and control groups. The mutant gene is a potential new clarification point regarding recurrent spontaneous abortion in several cases.

Key words: COX-2, Gene, Recurrent spontaneous abortion, Prostaglandin, DNA, PCR

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INTRODUCTION

Recurrent spontaneous abortion is commonly defined as the loss of three or more consecutive pregnancies prior to 20 – 28 weeks. Epidemiologically, it affects up to 5% of fertile couples. Studies indicate that the risk of subsequent pregnancy loss is approximately 24% for the next two clinical pregnancies, while it is 30% for the next three, 40% for the next four sequential spontaneous abortions, and that it affects an even higher proportion of women who are aged 35 years old or more [1,2].

The cyclooxygenase enzymes catalyst is a key factor in the transformation of arachidonate to prosglandins. There are two COX isoforms which vary fundamentally in type. Both iso-enzymes transform arachidonic acid prostaglandins but differ in their distribution and physiological functions. COX-1 is extracted in generality tissues whereas COX-2 ordinarily is absent but is activated by numerous physiologic stimuli. COX-1 and COX-2 also have differences in terms of mRNA splicing, stability, and translational efficiency [3,4].

Cox-2 is a biological macromolecule that is essential for mammalian blastocyst implantation and it plays a critical role in phase III after implantation. It is expressed in endometrial epithelial cells and stromal cells during blastocyst implantation. Prostaglandin also plays a vital role in embryonic circulation. COX-2 deficiency results in defective ovulation, fertilization, and decidualization [5–7].

The cyclooxygenase-2 gene in humans is present on chromosome 1 (1q25.2-q25.3), entity 8.3kb in length, and involves 10 exons. Numerous SNPs in the promoter region of the COX-2 gene have been described and connected with lower promoter activity in vitro. Generally the polymorphisms of the COX-2gene influence the transcription average, prostaglandin pro-

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duction, and are associated with implantation failure[8].

There are different results in the studies undertaken worldwide involving gene polymorphism and its relationship with recurrent spontaneous abortion. In Sudan, there has been published data on this subject that does not involve the proposal of gene polymorphism. This study was designed to determine whether there was possible cyclooxygenase-2 gene polymorphism among Sudanese women with a record of recurrent spontaneous abortion.

METHODS

A descriptive analytical case-control hospital-based study was carried out at the national center of neurological sciences in the research laboratory in Khartoum, Sudan during the period from April to September 2021. Each patient came to the obstetrics and gynecology department at Ibrahim Malik teaching hospital and was diagnosed with recurrent spontaneous abortion (more than three recurrent abortions) during the mentioned period, and these individuals were included as cases until the sample reached (44). Apparently healthy (50) women with no history of abortion and without any other risk factors related to abortion were selected as the control group. Women diagnosed with any disease such as SLE, lupus anticoagulant, CMV, and toxoplasma that can cause recurrent abortions were excluded from the study.

The participants at Ibrahim Malik hospital were selected using the convenience sampling technique (non-probability) until the sample size (n = 94) was obtained. Furthermore, the subjects were interviewed using questionnaires. From each of the subjects, 3 ml of venous blood was gathered in sterile containers with Ethylene Diamine Tetra-acetic Acid (EDTA). The DNA was isolated using the standard phenol chloroform extraction method. Conventional PCR was using to amplify the COX-2 gene. The primers were designed using the Prime3 software. The forward primer for COX-2 was designed as "5- TGGCCATCAATGGTACTGAA -3"and reverse as "5-CGGGAATTGCATCTGTTT -3" with a product size of 230 bp fragment.

Polymerase chain reaction - PCR

Specifically, 14 μl of ddH2O was placed in a PCR tube, then 4 μl of master mix, 1 μl of forward primer, 1 μl of reverse primer, and 2 μl of DNA sample were added and then vortexed. The PCR tube containing this mixture was placed in a thermal cycler machine (SwiftTM MaxPro SWT-MXP-BLC-4) at the following condition: denaturation temperature 94^{o}C for 30 secs, annealing temperature at 58^{o}C for 30 sec, and extension temperature at 72^{o}C for 30 sc. The last elongation was adjusted for 10 minutes at 72 °C. The PCR reaction was set at 35 cycles.

Gel-Electrophoresis

The products of the PCR were separated on agarose gel (0.7 g of agarose gel + 28 ml of DW + 7 ml T.E buffer; this mixture was placed in a microwave for 1 minute and then 1 μl of Ethidium Bromide was added and mixed. It was then poured onto a Gel-Electrophoresis tray and left to dry). After it dried, the PCR amplification products and 100-base-pair DNA ladder were used to fill in the wells, and then the running buffer was poured and ran at 150V for 16 minutes. The results were trans-illuminated with UV light and visualized through a gel documented system. The PCR products were dispatched for sequencing to Macrogen Europe Laboratory.

The data was entered and organized into a Microsoft Office Excel 2010 data sheet, then for the analysis, SPSS version 23 was used. The sequencing results were analyzed by employing various bioinformatics soft-wares and tools. The obtained sequences were aligned using the BioEdit- ClustalW software via a normal sequence from GenBank (National Center of Biotechnology Information, Accession Number NC_000001.11). Following this, they were examined for the presence of polymorphisms.

Ethical approval for this study was acquired from the Ethical Review Board in the Faculty of the Medical Laboratory of the University of Medical Science and Technology. The participants were fully informed of the advantages and disadvantages (oral informed consent) before participating.

RESULTS

Demographic data

A total of 94 Sudanese women were enrolled in this study to detect COX-2 polymorphism from the setting of Ibrahim Malik Teaching Hospital during the study period. Conclusively, 44 (46.8%) of the patients with recurrent spontaneous abortion were aged from 20 to 43 years old (mean 32.14; SD6.374). Additionally, 50 (53.2%) of the clearly healthy women were selected as the control group and their age ranged from 18 to 46 years old (mean 26.28; SD 6.52480) (**Table 1**) (**Figures 1 and 2**). The results reveal that the most affected age group was the women aged between 35 – 40 years old. The highest frequency for the abortion number was where most of them had aborted three times with a frequency about 77.3 %, four times about

Table 1: Basic characteristic of study population

Parameters	No (%)	Mean
Sex	Female	
Case	44 (46.8%)	32.14 SD 6.374
Control	50 (53.2%)	26.28. SD 6.52480
Age (cases)		
18-24	6 (13.6%)	
25-34	16 (36.4%)	
35-45	15 (34 .1%)	
>40	7 (15.9%)	

Table 2: Distribution of variables

Number of abortion	3	34	77.3
	4	4	9.1
	5	1	2.3
	6	3	6.8
	9	1	2.3
	Total	44	100.0
Gestational age	6 weeks	8	18.2
	7-10 weeks	15	34.1
	11-14 weeks	21	47.7
	Total	44	100.0
Smoking	Yes	1	2.3
	No	43	97.7
	Total	44	100.0
Genetic disease	Yes	5	10.0
	No	39	88.6
	Total	44	100.0

Table 3: Association between the presence of COX-2 gene polymorphism in the case and control

		PCR result		Total	p-value
		positive	Negative		
Cases	Count	44	0	44	0.001
	Expected Count	36.5	7.5	44.0	
Controls	Count	34	16	50	
	Expected Count	41.5	8.5	50.0	

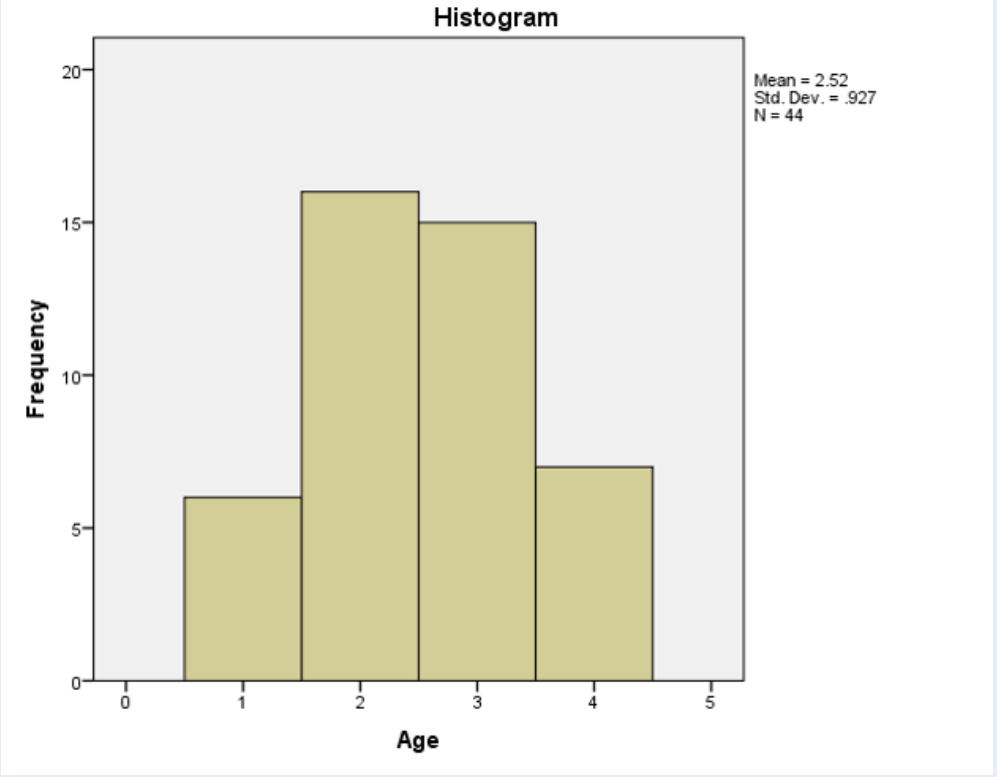

Figure 1: Disturbution of patient according to the age.

9.1%, five times 2.3%, six times 6.8%, and nine times about 2.3%. The frequency of gestational age was 11-14 weeks for about 47.7%, 7-10 weeks about 34.1%, and 6 weeks 18%. Regarding the risk factors for the recurrent miscarriage; about 2. 3% were smokers, 10% had a history of genetic disease, and in terms of treatment, only about 2.3 % had taken antibiotic, heparin, thyroxien, and vitamins respectively. All of them had taken folic acid (**Table 2**).

Molecular study

In the current research, 230 bp of the C0X-2 gene was revealed using gel electrophoresis after PCR (**Figure 3**). The results of the detected gene for all subjects were analyzed. The X2-test points out that there were significant differences in the gene expression and distribution between the case and control groups (p value 0.001) (**Table 3**).

Sequencing Results

The sequencing results were aligned with the reference sequence of the COX-2 gene (accession number NC_000001.11 in NCBI). The analysis showed that there was a change of C>A at Location 1:633149 (Rs1481253765) and a change of G> T at cDNA position 427, Location 1:633183 (Rs1232472838) (**Figures 4 and 5**). These changes were detected in 25% of the samples.

DISCUSSION

Recurrent spontaneous abortion is the health issue that impacts about 10.5% of women of child-bearing age. While large scale studies have specified different factors such as genetics, endocrine factors, autoimmune, and infections as affected approximately 50% of patient with RSA, the other 50% remains unexplained. Recent data supplies evidence that COX-2 has a critical role in molecular implantation mechanisms[5].

Our research hypothesis was based on the evidence the imponderables in COX-2 gene involved a risk factor for RSA, therefore this study aimed to detect cyclooxygenase-2 gene polymorphisms among Sudanese women with recurrent spontaneous abortion. It was found that when comparing between the case and control groups regarding the COX-2 allele gene, there was a significant difference between the two groups (p value 0.001). This finding disagrees with the

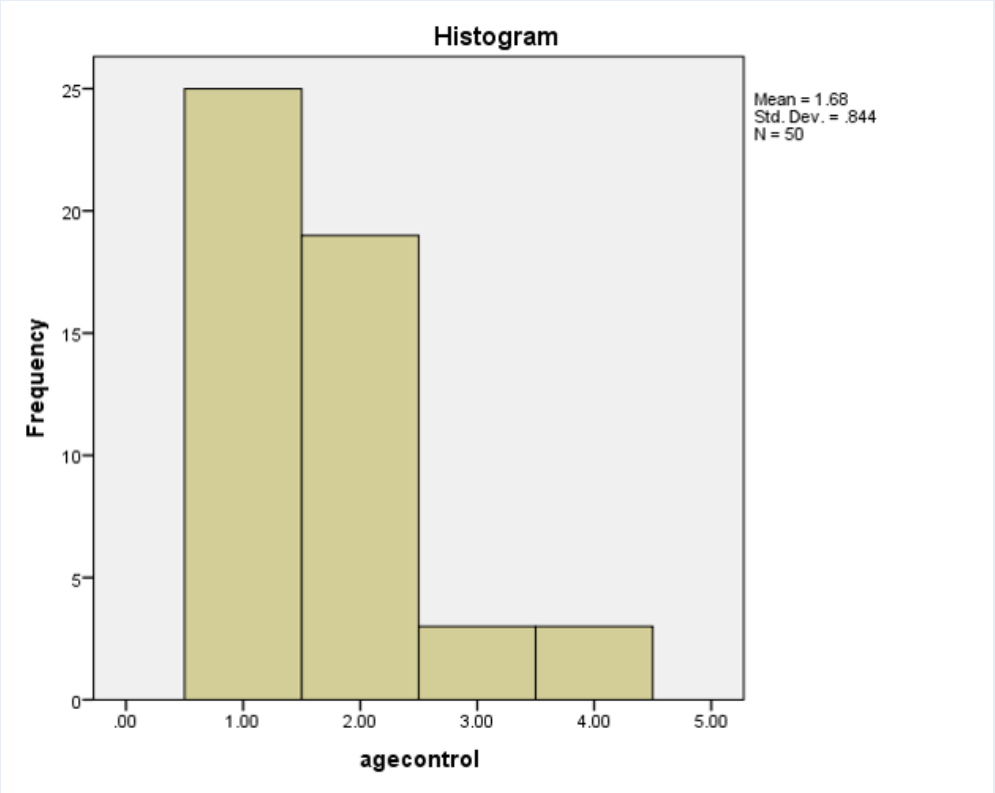

Figure 2: **Distribution of healthy control according to age.**

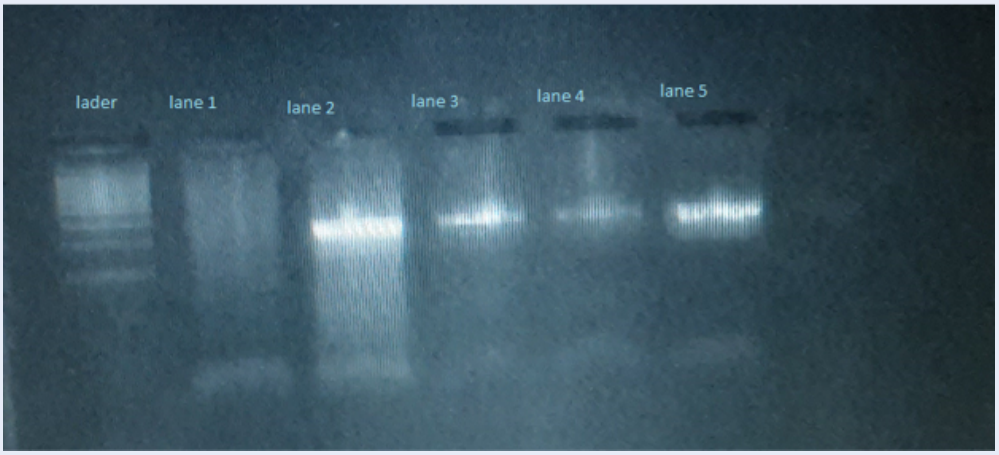

Figure 3: **Gel Electrophoresis for COX-2 gene**
Lane 1 (100 bp ladder), lane 2, 3,4,5,6, and 47(230 bp product of COX-2 gene)

Figure 4: **Bio-Edit clustal W results for cases group with reference gene sequence of COX-2 gene.**

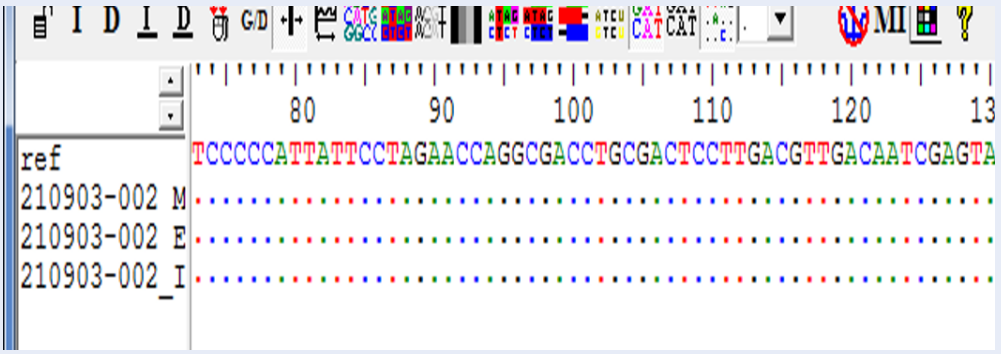

Figure 5: **Bio-Edit clustal W results for controls group with reference gene sequence of COX-2 gene**.

work of Fu Hua et al. who reported that the COX-2 expression of the experimental group was significantly lower than the control group. Another study in China done by Wang, Zhao, and Lin in 2010 using RT-PCR found that the mRNA expression level of COX-2 was significantly lower than that of the control group[5]. Another study reported that the significantly higher production of COX-2 during the implantation window in the endometrial tissue of some of the sample women may be attributed to the increased expression of proinflammatory cytokines IL-1b, TNF-a, IFN-g, and TGF-b1[9]. In addition, Singh et al. also reported that COX-2 expression was higher in relation to recurrent spontaneous abortion[10].

In the present study, regarding the Sanger sequences analysis, the data shows that the patients carrying the A allele for C>A and the T allele for the G>T variants of the C0X-2 gene have a significantly higher risk of abortion when compared to women with wild type allele. This may relate to the testimony on the important of the C0X-2 C>A and G>T variants as molecular biomarkers of RSA in Sudanese women. This result might raise the risk of recurrent abortion in women carrying this variant according to the hypothesis that a functional variant in the COX-2 gene may change the molecular implantation mechanisms. Salazar et al. reveled that women carrying the C allele for the −765G>C variant of the COX-2 gene had a significantly higher risk of implantation failure than women with the wild type allele[8].

Due to the language barrier and gender differences, the sample collection was challenging. Many patients were not willing to give a sample of their blood. The time for the sample collection was limited as not many patients with RSA were coming to the Ibrahim Malik Teaching Hospital.

CONCLUSIONS

This study was conducted to detect the presence of COX-2 gene polymorphisms in Sudanese patients with RSA. Our data indicates that, for the first time, that COX-2−C>A and G>T polymorphism is related to recurrent spontaneous abortion. This observation is necessary to validate in a much larger population.

ABBREVIATIONS

CMV: Cytomegalovirus

NCNS: National Center Of Neurological Sciences

RSA: Recurrent Spontaneous Abortion

SLE: Systemic Lupus Erythematosus

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medical science and Technology for their helpful and assistance.

AUTHOR'S CONTRIBUTIONS

All authors equally participated in this manuscript, implicated wrote, corrected and approved this manuscript.

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None.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the amended Declaration of Helsinki. The institutional review board approved the study, and all participants provided written informed consent.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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