



# The Potential Role of Reactive Oxygen Species Produced by Low-Density Neutrophils in Periodontitis

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## Abstract

**Objective** Neutrophils own an arsenal of dischargeable chemicals that enable them to handle bacterial challenges, manipulating innate immune response and actual participation in acquired immunity. The reactive oxygen species (ROS) are one of the most important chemicals that neutrophils discharge to eradicate pathogens. Despite their beneficial role, the ROS were strongly correlated to periodontal tissue destruction. Lowdensity neutrophils (LDN) have been recognized for producing enhanced quantities of ROS. However, the potential role of ROS produced by LDN in periodontitis is unknown. The aim of the study was to investigate the impact of ROS produced by LDN in periodontal diseases.

**Materials and Methods** Venous blood and periodontal parameters were obtained from 100 systemically healthy subjects divided into 40 participants with healthy periodontium in the control group and 60 with unstable periodontitis in the study group. Flow cytometry was used to measure the production of ROS by LDN in both groups.

**Statistical Analysis** The data were analyzed for normal distribution using the Shapiro-Wilk test at  $p < 0.05$ , Spearman's correlations, and Mann-Whitney U test. Statistical analysis was performed in SPSS v25.

**Results** No difference between the groups had been obtained in ROS production by LDN. However, a significant positive correlation existed between ROS and clinical attachment loss in periodontitis.

**Conclusion** LDN exhibits the same ROS generation capacity in the control and periodontitis groups.

## Keywords

- ▶ periodontitis
- ▶ reactive oxygen species
- ▶ low-density neutrophils

## Introduction

Periodontitis is a multifactorial progressive condition characterized by continuous destruction of periodontal tissue under the burden of a dysbiotic microflora, host immune response, environmental factors, and subject genetic suscep-

tibly.<sup>1,2</sup> Periodontitis encompasses the existence of inflammation in the periodontal tissues, the development of periodontal pockets, a breakdown of connective tissue, alveolar bone loss, and potential tooth loss.<sup>3-7</sup> Despite their beneficial role in eradicating pathogens, inflammation is a double-edged sword; it is considered a crucial source of

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periodontal tissue destruction, involving leukocytes, complement, and reactive oxygen species (ROS).<sup>8</sup> ROS seem to be short-lived, extremely reactive oxygen-reduced radicals, including superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), the hydroxyl radical (OH), and singlet oxygen.<sup>9</sup> At the cellular level, ROS is crucial for eukaryotic cell physiologic functions such as signaling transmission, differentiation, apoptotic cell death,<sup>8,9</sup> and pathogen oxidative death.<sup>10</sup>

A clinical study revealed that serum reactive oxygen metabolites were positively correlated with immunoglobulin G antibodies to keystone periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetem-comitans*, and *Prevotella intermedia*.<sup>11,12</sup>

Furthermore, the level of ROS markedly regulates many biological events of most cellular components in the periodontium. For instance, a reduced ROS level decreases bone loss by downregulating the osteoclast differentiation marker genes; conversely, it enhances the proliferation and differentiation of human periodontal ligament fibroblasts (hPDLFs) and osteoblasts.<sup>13</sup>

Low-density neutrophils (LDN) are a unique attention-seeking neutrophil phenotype strongly associated with various immunological diseases, endocrine disorders, systemic diseases, and infections.<sup>10</sup> Based on previous studies on healthy subjects, the LDN exhibits enhanced ROS-generating capacity compared to the normal-density neutrophil.<sup>10</sup>

To the best of our knowledge, the potential impact of ROS produced by LDN in the periodontitis has not been studied yet. However, this study tries to illustrate this role in the pathogenesis of periodontitis using a multicolor flow cytometry.

## Materials and Methods

### Study Design

This observational research was conducted from August 2022 to March 2023 at the College of Dentistry, University of Baghdad.

### Ethical Approval

All procedures used in this study adhered to the guidelines outlined in the Helsinki Declaration of 1964 and its subsequent revisions, specifically concerning research involving human subjects. The protocol for this study has been authorized by the Ethics Committee of the College of Dentistry at the University of Baghdad. The reference number for this approval is 450, the project number is 450622, and the approval date is January 19, 2021. Following the provision of comprehensive information elucidating the nature and objectives of the research, every participant was requested to affix their signature on an informed consent document. Following the participants' agreement, a clinical examination was conducted, and then blood samples were collected from each individual.

### Inclusion Criteria

One hundred systemically healthy participants were allocated into two groups:

- The control group consisted of 40 subjects with healthy periodontium defined as bleeding on probing (BOP) less than 10% and periodontal pocket depth (PPD)  $\leq 3$  mm. Additionally, there was no evidence of attachment loss during periodontal probing.<sup>14</sup>
- The periodontitis group consisted of 60 subjects with unstable periodontitis, defined as BOP greater than 10%, with clinical attachment loss (CAL) at the interproximal area at  $\geq 2$  nonadjacent teeth or two teeth with buccal or oral CAL of 3 mm with pocketing greater than 3 mm.<sup>15</sup>

### Exclusion Criteria

Patients with dental implants, systemic or oral autoimmune illness, infections, inflammatory diseases, drug intake (antibiotics, nonsteroidal anti-inflammatory drugs) within 3 months, endocrine disorders, systemic problems, pregnancy, and smoking habits were excluded. The wisdom teeth were excluded from periodontal examinations.

### Subjects Eligibility

Patients with periodontitis referred for periodontal therapy were initially screened ( $n = 113$ ) to evaluate their eligibility for recruitment. After applying the inclusion/exclusion criteria, 53 patients were excluded for different reasons, and 60 patients were included in the final analysis. Later, the patients with healthy periodontium were included ( $n = 40$ ) as controls (→ Fig. 1).

### Clinical Examination

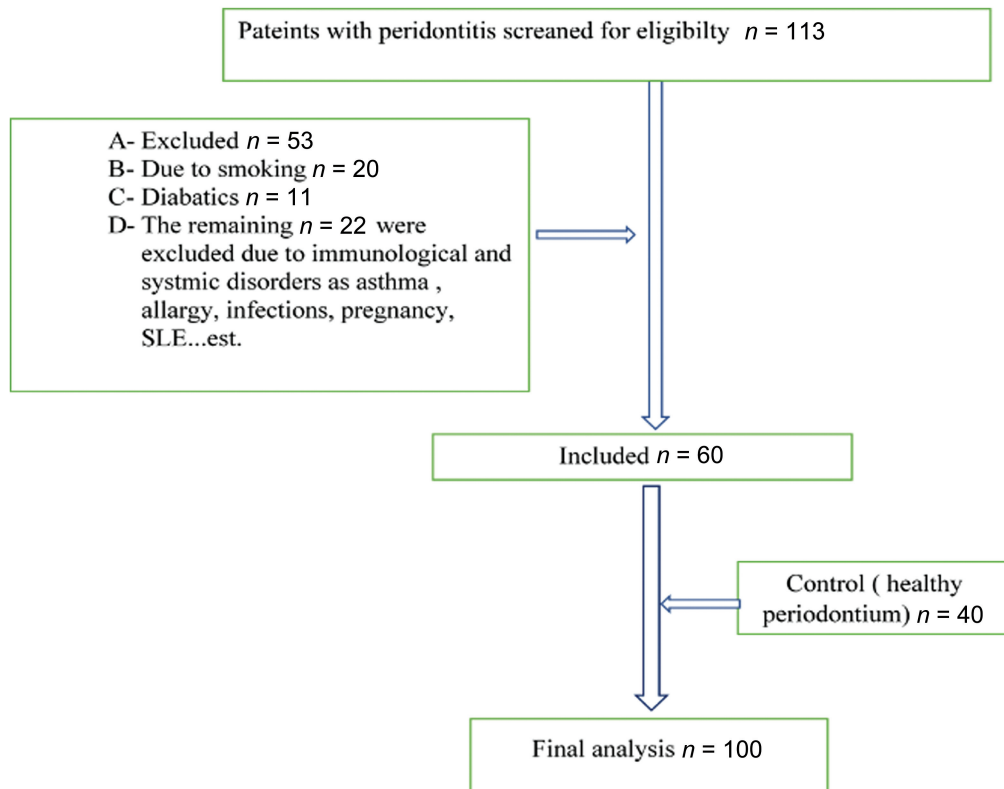
All clinical examinations were done utilizing the Michigan O probe (Osung USA, Houston, United States) with markings at 1, 2, 3, 5, 7, 8, 9, and 10 mm. Six surfaces were examined to estimate BOP%, PPD, and CAL: mesiobuccal, middle buccal, distobuccal, mesio-oral, middle oral, and disto-oral. In contrast, plaque index (PI)<sup>16</sup> was estimated by examination of four surfaces (mesial, distal, buccal, and oral).

### Blood Collection

After clinical examination and subject allocation, 5 mL of intravenous blood was collected and placed in a 10-mm ethylenediaminetetraacetic acid (EDTA) tube. The collected blood was preserved at 4°C protected from light for 30 minutes. LDN purification must be done within a period not exceeding 6 hours to purify pure, healthy, and activated LDN.<sup>10</sup>

### Purification of LDN

The LDN were purified by mixing 5 mL of anticoagulated blood with 2 mL of 6% dextran T500 by inversion and allowing it to rest for 45 minutes to precipitate red blood cells. Next, the layer of leukocyte-rich plasma was placed carefully on top of 5-mL Lympho-Paque at 1.077 g/mL density. The sample was centrifuged at 520 g for 20 minutes at 4°C using a Thermo Scientific Megafuge 8R Small Benchtop Centrifuge. A layer of mononuclear cells (MNC), including monocytes, lymphocytes, and LDNs, is located at the plasma-lympho-paque interface (→ Fig. 2). LDN-containing MNC was collected, diluted 1:2 with phosphate buffer saline (PBS), and stored in ice until use.

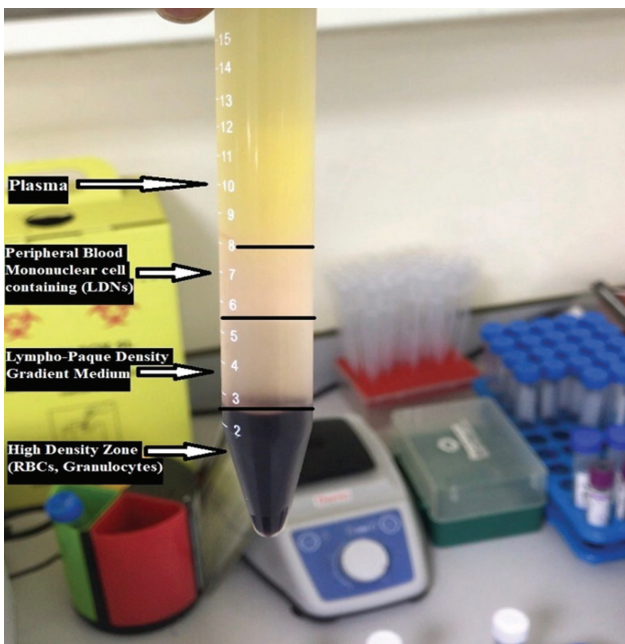


**Fig. 1** Subject eligibility chart flow.

### Measurement of Reactive Oxygen Species

ROS production was measured by detecting fluorescence changes in LDNs loaded with dihydrorhodamine 123 (DHR-123). Cells ( $1 \times 10^6$ ) were suspended in 100 mL of 15 mM DHR-123 in PBS and incubated for 15 minutes at 37°C in the dark. Cells were washed with 1 mL PBS, resus-

ended in 100 mL of PBS containing 20 nM of Probol-12-myristate-13-acetate (PMA), and incubated at 37°C in the dark for 50 minutes. Next, cells were washed in cold PBS and resuspended in 1% paraformaldehyde in PBS. Finally, cells were kept in a cold and dark place until analyzed by flow cytometry. The cells were gated by a dot-plot analysis, and 10,000 cells were acquired per sample. The cells were analyzed in BD FACSCanto II multicolor flow cytometry with the BD FACSDiva Software v9.0 (►Fig. 3).



**Fig. 2** Purification of low-density neutrophils (LDN) in peripheral blood mononuclear cells (PBMCs).

## Results

### Descriptive Data

In the control group, the descriptive data show the minimum, maximum, mean, and standard deviation of different variables.

The descriptive data in the periodontitis group showed the minimum, maximum, mean, and standard deviation of different variables (as shown in ►Table 1).

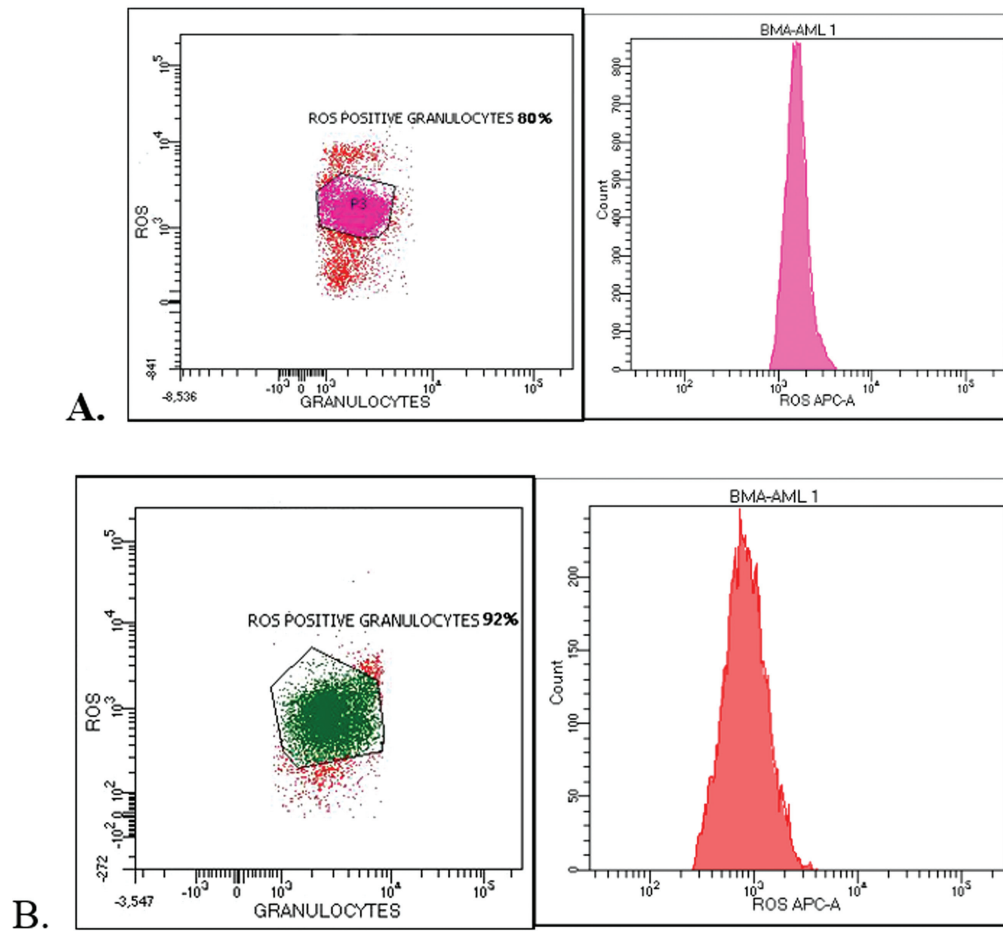
**Intergroup comparisons** showed a significantly higher BOP and PI in the periodontitis group than in the control group ( $p < 0.01$ ) (as shown in ►Table 2).

However, no significant difference was observed in ROS production between the two groups (as shown in ►Table 3).

### Correlations

#### Correlations in the Control Group

The observed data shows a significant positive correlation between BOP% and PI in the control group. However, no



**Fig. 3** Reactive oxygen species (ROS) expression via LDN in (A) a healthy periodontium. (B) Periodontitis.

correlation was observed between ROS and the other variables in the control group (► **Table 4**).

**Correlations in the Periodontitis Group.**

The collected data show a significant positive correlation among all periodontal parameters; however, ROS was significantly and positively correlated with CAL in the periodontitis group ( $p = 0.275$ ; 2-tailed = 0.034) (► **Table 5**).

**Discussion**

Neutrophils are the predominant leukocytes in circulation and are considered the main cellular arm of innate immunity, which responds to inflammation and infection.<sup>17</sup> In inflamed tissues, neutrophils perform several antimicrobial tasks,<sup>18</sup> such as degranulation,<sup>19</sup> ROS generation,<sup>20</sup> phagocytosis,

and neutrophil extracellular trap (NET) formation.<sup>21</sup> In addition to innate immunological activities, neutrophils modulate adaptive immune responses.<sup>22</sup> In fact, neutrophils do not appear to be a homogeneous population that often behaves similarly; however, multiple subpopulations of neutrophils have been proposed in diverse health and disease situations.<sup>23</sup>

LDN is a unique neutrophil phenotype that exists in large numbers in various pathological disorders like inflammations, infections, cancer, and immunosuppression.<sup>24</sup> Based on a previous study, LDN has also been detected in the peripheral blood of systemically healthy subjects and exhibits an enhanced capacity for ROS compared to the normal high-density neutrophil (HDN).<sup>10</sup>

Interestingly, the outcomes of our study revealed no significant difference in ROS produced via LDN between

**Table 1** Descriptive data in the control group

	Minimum	Maximum	Mean	Std. deviation
Age (Year)	21	55	34.5	±9.94
BOP %	0.021	0.091	0.0682	±.0227
PI (%)	0.111	0.511	0.37171	±.115251
ROS	0.44	0.99	0.8498	±.14648

Abbreviations: BOP, bleeding on probing; PI, plaque index; ROS, reactive oxygen species.

**Table 2** Descriptive data in the periodontitis group

	Minimum	Maximum	Mean	Standard deviation
Age (Year)	21	57	36.28	±9.372
BOP %	0.111	0.881	0.405	±.2247
PI (%)	0.31	2.5	1.28	±.57
PPD (mm)	4.1	9.1	5.51	±1.279
CAL (mm)	1.1	11.1	5.26	±2.6670
ROS	0.410	0.99	0.89268	±.111128

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PI, plaque index; PPD, periodontal pocket depth; ROS, reactive oxygen species.

**Table 3** The intergroup comparison

Biomarker	Mean control	Mean periodontitis	p-value*
Age (Year)	34.48	36.28	0.430 (NS)
BOP %	0.0682	0.405	0.000*
PI (%)	0.37171	1.28	0.000*
ROS	0.8498	0.89268	0.320 (NS)

Abbreviations: BOP, bleeding on probing; NS: non significant at  $P$ -value  $\geq 0.05$ ; PI, plaque index; ROS, reactive oxygen species.

\*significant at  $P$ -value  $< 0.05$ .

**Table 4** The correlations in the control group

	Spearman correlation	Age	BOP %	PI	ROS
Age	Correlation coefficient	1.000			
	Sig. (2-tailed)				
BOP %	Correlation coefficient	0.052	1.000		
	Sig. (2-tailed)	0.748			
PI	Correlation coefficient	-0.131	0.689 <sup>a</sup>	1.000	
	Sig. (2-tailed)	0.419	0.000*		
ROS	Correlation coefficient	0.004	0.094	0.238	
	Sig. (2-tailed)	0.979	0.565	0.139	

Abbreviations: BOP, bleeding on probing; NS: non significant at  $P$ -value  $\geq 0.05$ ; PI, plaque index; ROS, reactive oxygen species.

<sup>a</sup>Refers to the significant correlation coefficient.

\*significant at  $P$ -value  $< 0.05$ .

**Table 5** The correlations in the periodontitis group

	Spearman correlation	Age (Year)	BOP %	PI	PPD	CAL	ROS
Age (Year)	Correlation coefficient	1.000					
	Sig. (2-tailed)						
BOP %	Correlation coefficient	0.141	1.000				
	Sig. (2-tailed)	0.284					
PI (%)	Correlation coefficient	0.175	0.808 <sup>a</sup>	1.000			
	Sig. (2-tailed)	0.180	0.000				
PPD (mm)	Correlation coefficient	0.303 <sup>b</sup>	0.808 <sup>a</sup>	0.653 <sup>a</sup>	1.000		
	Sig. (2-tailed)	0.019	0.000	0.000			
CAL (mm)	Correlation coefficient	0.176	0.492 <sup>a</sup>	0.442 <sup>a</sup>	0.532 <sup>a</sup>	1.000	
	Sig. (2-tailed)	0.179	0.000	0.000	0.000		

(Continued)



**Table 5** (Continued)

	Spearman correlation	Age (Year)	BOP %	PI	PPD	CAL	ROS
ROS	Correlation coefficient	0.280 <sup>b</sup>	-0.015	0.094	0.160	0.275 <sup>b</sup>	
	Sig. (2-tailed)	0.030	0.908	0.473	0.222	0.034	

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PI, plaque index; PPD, periodontal pocket depth; ROS, reactive oxygen species.

<sup>a</sup>Refers to the significant correlation coefficient.

<sup>b</sup>Refers to the Sig. (2-tailed).

the study groups, which may indicate that LDN is an excellent ROS producer regardless of the periodontal status. Despite this, LDN exhibits more ROS production capacity in the periodontitis group at 89% compared to the healthy control group at 85%. However, this may be due to the systemic dissemination of periodontal pathogens and their products via the ulcerated pocket epithelium.<sup>25,26</sup>

Additionally, a significant positive correlation was observed between ROS and CAL in the periodontitis group. Based on this outcome in healthy subjects, ROS is beneficial in pathogen eradication and differentiation of cells.<sup>11</sup> On the other hand, in periodontitis patients, increasing ROS coincided with increasing destruction of the periodontal tissue (CAL). In conclusion, enhanced ROS benefits the healthy periodontium; in contrast, it is harmful in periodontitis.

However, the schizophrenic behavior of ROS in the current study could be related to the homeostatic imbalance between ROS and antioxidant defense systems,<sup>27,28</sup> as the periodontitis patients showed lower plasma and serum total antioxidant concentrations (TAOC) levels than healthy controls.<sup>28,29</sup>

The TAOC is the main body defense system for neutralizing ROS. Furthermore, decreased TAOC and elevated ROS might be risk factors for periodontitis or might be induced by periodontal inflammation.<sup>30</sup> However, to estimate the precise effect of TAOC, the analysis of site-specific LDNs purified from saliva, gingival crevicular fluid (GCF), and the junctional epithelium is mandatory. Indeed, in the murine model, the LDN can be characterized by the expression of CD11b and Ly6G+ markers.<sup>31</sup> However, in humans, the absence of Ly6G+ antigen that distinguishes normal-density neutrophils (NDN) from LDN makes site-specific studies with high overlap effects between these neutrophil subpopulations.<sup>32-34</sup> Consequently, the density gradient centrifugation of peripheral blood is the sole method for the purification of LDN. The above-mentioned considerations present a major challenge to accurately evaluate the actual impact of TAOC in neutralizing LDN ROS.

## Conclusion

Irrespective of the periodontal status, LDN demonstrates an increased capability for generating ROS. The enhanced ROS benefits the healthy periodontium; conversely, it is harmful in periodontitis.

### Conflict of Interest

None declared.

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