Article

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Dysregulated Antioxidant Network and Increased iNOS Signaling in Platelets of Children with Autism Spectrum Disorder

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Background: Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by the significant involvement of both environmental and immunological factors. The pathophysiology of ASD has been linked to dysregulation of the antioxidant network and production of oxidants in immune cells. Previous studies have demonstrated disequilibrium in different enzymatic antioxidants in the plasma, red blood cells, and leukocytes of individuals with ASD; however, there has been no investigation thus far into the evaluation of these antioxidants in peripheral platelets in both individuals with ASD and typically developing control (TDC) children.

Methods: Given this context, we investigated the levels and functions of key enzymatic antioxidants in peripheral platelets of TDC (n = 23)/ASD (n = 26) individuals, including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and thioredoxin reductase (TRxR) through flow cytometric and enzymatic activity analyses. Further, levels of oxidative stress were evaluated by analysis of inducible nitric oxide synthase (iNOS) and nitrotyrosine formation in the platelets of both groups.

Results: Our findings reveal a marked reduction in SOD1 ($p < 0.0001$) and TRxR1 ($p < 0.01$) expression in the platelets of ASD individuals, as evidenced by diminished SOD1+ and TRxR1+ immunostaining in CD42+ cells. SOD ($p < 0.01$) and TRxR ($p < 0.01$) activity were also significantly lower in ASD participants compared to the TDC group. In contrast, when comparing individuals with ASD to TDC group, GPx/GR activity/expression in platelets is either decreased or unaffected. A notable increase in iNOS ($p < 0.0001$) coupled with reduced SOD/TRxR activity in ASD platelets correlated with a significant rise in nitrotyrosine expression ($p < 0.001$), indicative of oxidant damage.

Conclusions: Our findings demonstrate, for the first time, that ASD individuals have a disrupted enzymatic antioxidant system and heightened oxidative stress in their peripheral platelets. This imbalance in enzymatic antioxidants may significantly impact the development of ASD and its associated comorbidities.

Keywords: ASD; enzymatic antioxidants; TRxR; iNOS; oxidative stress; platelets

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental illness that occurs in infancy and includes a variety of clinical symptoms mostly associated with difficulties in sensory perception, cognition, communication, and socialization [1]. Individuals with ASD also exhibit aggressive behavior, lack of focus, self-harm tendencies, and heightened levels of stress [2]. ASD is typically more prevalent in males than females, with a ratio of approximately 4 to 1, as indicated by earlier studies [3,4]. While multiple studies have identified different environmental and genetic factors that may contribute to ASD, a conclusive etiology has not yet been discovered [3,5].

Oxidative stress has emerged as a strong candidate among the various variables that are implicated in the etiology of ASD [6,7]. Oxidative stress typically ensues from an overabundance of reactive oxygen species (ROS) produced by various cellular sources, including enzymatic and spontaneous sources [7–9]. A number of researchers have, in the past, examined a variety of indicators that are associated with oxidative stress in the blood, urine, and postmortem brain samples. When comparing individuals with ASD to healthy controls, these compartments have higher levels of oxidants and oxidative stress indicators. Nitrotyrosine, lipid peroxides/hydroperoxides, ROS, nitric oxide, chlorotyrosine, and isoprostanes are some of the oxidative

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

stress indicators that have been shown to be elevated in individuals who have ASD [7,10–12]. These biomarkers are often the consequence of direct or indirect assault by reactive oxidants generated by mitochondrial malfunction or by enzymes that create them such as inducible nitric oxide synthase (iNOS) [8,9]. Recent studies have shown that neutrophils and monocytes have elevated levels of iNOS-mediated oxidative stress in individuals with ASD [13,14]; however, iNOS signaling has not been determined in platelets of individuals with ASD.

Antioxidants help to neutralize oxidants generated by normal cellular metabolism in immune cells such as B cells, T cells, monocytes, and neutrophils. These antioxidants may be enzymatic or non-enzymatic, such as glutathione reductase (GR), glutathione peroxidase (GPx), thioredoxin reductase (TRxR), superoxide dismutase (SOD), vitamin E, and vitamin C. A number of studies have shown that an overabundance of oxidants may cause enzymatic and non-enzymatic antioxidants to be depleted or altered [8,15–17]. Previous researches have shown that both enzymatic and non-enzymatic antioxidants undergo changes in both the blood and brain of individuals with ASD [12,18–20]. According to many investigations [7,19,21–23], altered enzymatic antioxidants, such as SOD, GR, and GPx, have been found in serum, red cells, neutrophils, monocytes, and the brain. Nonetheless, no previous research has examined the expression or activity of enzymatic antioxidants in peripheral platelets of individuals with ASD.

In light of this, we conducted an analysis of the primary enzymatic antioxidants and oxidant-generating enzyme, iNOS in the platelets of individuals with ASD. Our work demonstrates that enzymatic antioxidants such as SOD and TRxR are decreased in peripheral platelets of individuals with ASD. Dysregulation in enzymatic antioxidants is linked to elevation in iNOS and nitrotyrosine expression, indicating that peripheral platelets from individuals with ASD experience greater oxidative stress than those in the TDC group.

Materials and Methods

Participants

This cross-sectional research included 49 participants; 26 of those participants were children ASD, while the remaining 23 served as typically developing control (TDC). The ASD group consisted of 26 males with an average age of 6.78 ± 2.68 years (mean ± SD). In comparison, the TDC group contained 23 males with an average age of 6.35 ± 2.36 years (mean ± SD). The participants in the ASD group were selected from the Autism Research and Treatment Center, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. The children in the TDC group were selected from the Well Baby Clinic at King Khalid University Hospital, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. The existence or past of neurological or neuropsychiatric conditions (such as cerebral palsy, bipolar disorder, seizures, or tuberous sclerosis), metabolic disorders (such as phenylketonuria), or autoimmune/inflammatory conditions were the exclusion criteria for the ASD group. Children from TDC group were excluded if they had linguistic impairment, intellectual incapacity, neurological illnesses, inflammatory disorders, autoimmune disorders, or any known genetic condition. Children in both groups were mobile and in good health at the time of blood collection; none of them were taking any immune-modifying medications or vitamin supplements. All children who participated in this research had their written permission signed by their parents or legal guardians. The local ethical committee at Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia granted approval (IRB Approval number: E-10-220) for this study according to the national guidelines for research on human samples as stated in Declaration of Helsinki.

Clinical Assessment and ASD Diagnosis

The research evaluated ASD patients by conducting clinical examinations, reviewing their medical history, and performing neuropsychiatric assessments administered by highly skilled clinicians. Individuals with ASD met the diagnostic criteria for autism as outlined in the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders, published by the American Psychiatric Association in 2013 [24]. In addition, the severity of the condition was evaluated using the Childhood Autism Rating Scale (CARS), which measures a child's level of impairment on a scale of 1–4 in 15 specific domains with a maximum score of 60, as previously reported [25]. A child was diagnosed to have ASD if he had a score of greater than 30. ASD children with CARS score between 30–36 were categorized as moderate (n = 17), while ASD children with CARS score between 37–60 were categorized as severe (n = 9).

Isolation of Platelets from Peripheral Blood

In the morning, a sample of peripheral venous blood was obtained using a Vacutainer tube containing acid-citrate dextrose (364606, BD Biosciences, Franklin Lakes, NJ, USA). In order to prepare the children for venipuncture, children were kept occupied with play to reduce anxiety. Isolation of platelet rich plasma (PRP) from blood was done soon thereafter. The PRP was collected using centrifugation at 250 g for a duration of 15 min, resulting in the separation of the upper layer containing PRP and the lower layer consisting of red blood cells. To prepare the PRP for *in vitro* enzymatic tests, the top layer containing PRP was moved to a separate tube and spun once more at 1000 g for 20 min to obtain a platelet pellet. The pellet obtained was suspended in a ready-to-use proprietary cell lysis reagent II (FNN0021; Invitrogen™, Carlsbad, CA, USA) for biochemical estimations.

Measurements of SOD, GPx, GR, and TRxR Activity

The activities of SOD (706002, Cayman Chemical, Ann Arbor, MI, USA), GPx (K762-100, Biovision, Milpitas, CA, USA), GR (K761-100, Biovision, Milpitas, CA, USA), and TRxR (K763-100, Biovision, Milpitas, CA, USA) in platelets were assessed following the instructions provided by the manufacturers (Cayman Chemical, Ann Arbor, MI, USA; Biovision, Milpitas, CA, USA). The results were quantified in terms of mU/mg protein or U/mg protein or nmol/min/mg protein.

Flow Cytometric Analysis

Immunostaining of intracellular proteins was performed on fresh blood samples obtained from ASD and TDC groups using flow cytometry, following the methodology outlined in previous studies [7,14]. In summary, blood platelets were subjected to immunostaining using fluorophore-conjugated monoclonal antibodies specifically targeting human CD42, i.e., FITC-CD42 (303903, Biolegend, San Diego, CA, USA), or APC-CD42 (303912, Biolegend, San Diego, CA, USA), or PE/Dazzle-CD42 (303922, Biolegend, San Diego, CA, USA), or APC/Cy7-CD42 (303920, Biolegend, San Diego, CA, USA). Following usual step of permeabilization and washing, blood platelets were exposed to monoclonal antibodies targeting human APC-SOD1 (sc-101523, Santa Cruz Biotech, Dallas, TX, USA), FITC-GPx1 (sc-133160, Santa Cruz Biotech, Dallas, TX, USA), PE-GR (sc-133245, Santa Cruz Biotech, Dallas, TX, USA), FITC-TRxR1 (sc-28321, Santa Cruz Biotech, Dallas, TX, USA), or APC-iNOS (sc-7271, Santa Cruz Biotech, Dallas, TX, USA), or FITC-nitrotyrosine (sc-32757, Santa Cruz Biotech, Dallas, Dallas, TX, USA) for the purpose of immunostaining intracellular proteins. Extracellularly or intracellularly immunostained platelets were then examined by counting 20,000 occurrences using the FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). All the events in FSC *vs* SSC plot (platelets and leukocytes) except the debris were gated in for analysis of different markers. Platelets were identified using fluorophore-conjugated antibody against platelet marker, i.e., CD42 as described above. Further, intracellular proteins in CD42+ cells were identified in double positive quadrants using CXP software (Version 2.0, Beckman Coulter, Brea, CA, USA) as described below in figure legends. The data are shown in the form of flow graphs, as previously described [7,14].

Statistical Analysis

The data were presented as the mean ± standard error of mean (SEM). The data were evaluated using one-way ANOVA followed by Tukey's multiple comparison post-hoc test. The threshold for statistical significance was established at $p < 0.05$ to determine differences between the groups. Normality was checked by Shapiro–Wilk test which showed normal distribution of data for all studied parameters. The statistical studies were conducted using the GraphPad Prism statistical software (version 10, GraphPad Software, San Diego, CA, USA).

Results

Decreased SOD Expression/Activity in the Platelets of ASD Individuals

In previous research, the focus was mostly on examining enzymatic antioxidants in plasma, erythrocytes and leukocytes. However, in this study, we specifically examined the levels of important enzymatic antioxidants in platelets. Findings of the current study showed a significant decrease in expression of SOD1 protein ($p < 0.0001$) in platelets of individuals with ASD, in comparison to TDC group (Fig. 1A,B). This decrease was reflected by a reduced percentage of double positive immunostaining of SOD1+ and CD42+ cells in the ASD group as compared to the TDC group. Moreover, there was a further significant decrease in SOD1+ expression in platelets with increasing severity as depicted by a significant difference ($p < 0.05$) between M-ASD and S-ASD groups (Fig. 1A,B). In addition, the assessment of SOD activity exhibited a trend similar to that was observed in SOD1 protein expression in platelets (Fig. 1C). This indicates downregulation of SOD expression and activity in individuals with ASD.

Decreased GPx/GR Activity in the Platelets of ASD Individuals

Within the enzymatic antioxidant network, GPx serves to neutralize lipid hydroperoxides caused by oxidative stress. Consequently, it was investigated whether GPx was also affected in platelets of TDC/ASD group. Results of this study showed that the expression of GPx1 at the protein level was unchanged in platelets of individuals with ASD compared to the TDC group (Fig. 2A), however, the activity of GPx was shown to be significantly ($p < 0.05$) reduced in individuals with severe ASD compared to the TDC group (Fig. 2B).

Thereafter, the subsequent goal was to evaluate the expression and activity of the GR in platelets from both groups. Observations obtained from this study showed that the expression of GR protein in platelets in individuals with ASD was not different from that of the TDC group (Fig. 3A). However, there was a significant decrease in GR activity ($p < 0.01$) in platelets of individuals with ASD, as shown in Fig. 3B. This indicates that the activities of GPx and GR are compromised and inefficient in the detoxification process in the platelets of individuals with ASD.

Decreased TrxR Expression/Activity in Platelets of ASD Individuals

Then the next goal was to evaluate the expression and activity of the TrxR in platelets from both groups as this is an important enzyme in detoxification of oxidant species.

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BIOLOGICAL REGULATORS
and Homeostatic Agents

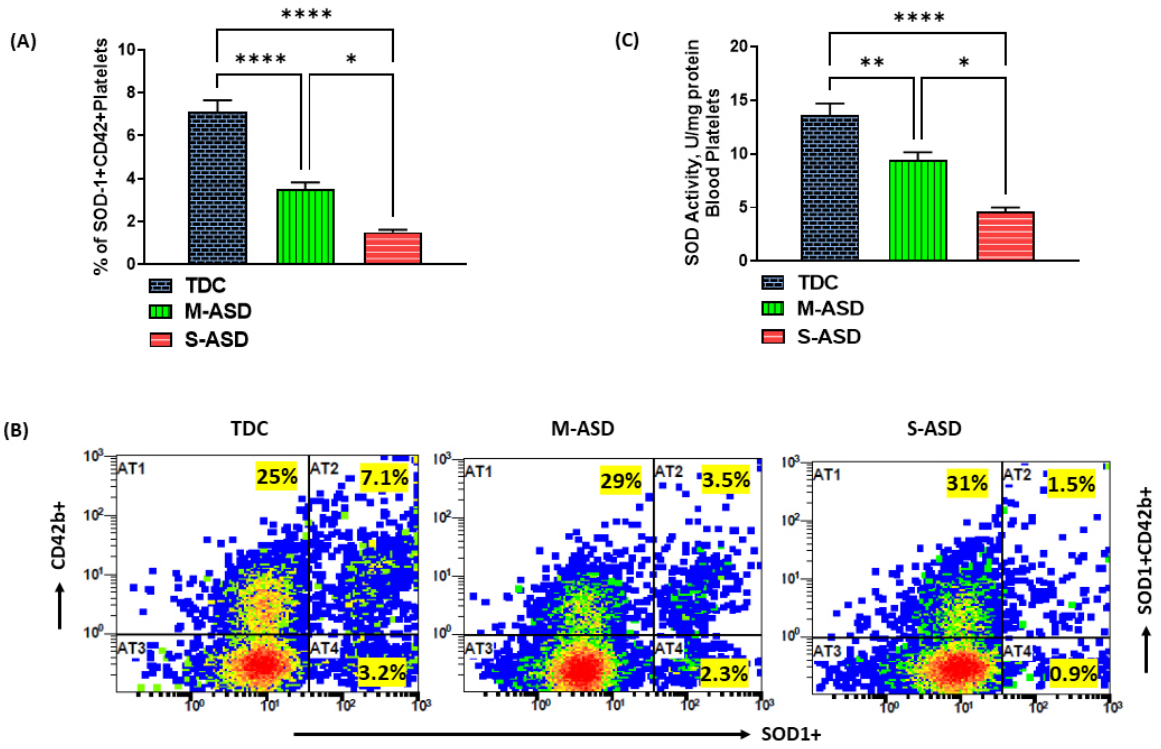

Fig. 1. Expression and activity of superoxide dismutase (SOD) in platelets of autism spectrum disorder (ASD) and typically developing control (TDC) subjects. (A) % of SOD1+CD42+ cells (AT2 quadrant of panel B is represented in this figure), (B) Flow plot showing double positive immunostaining of SOD1+CD42+ cells, and (C) SOD activity in platelets. Data are expressed as mean ± standard error of mean (SEM) (TDC group, n = 23; M-ASD group, n = 17; S-ASD group, n = 9). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

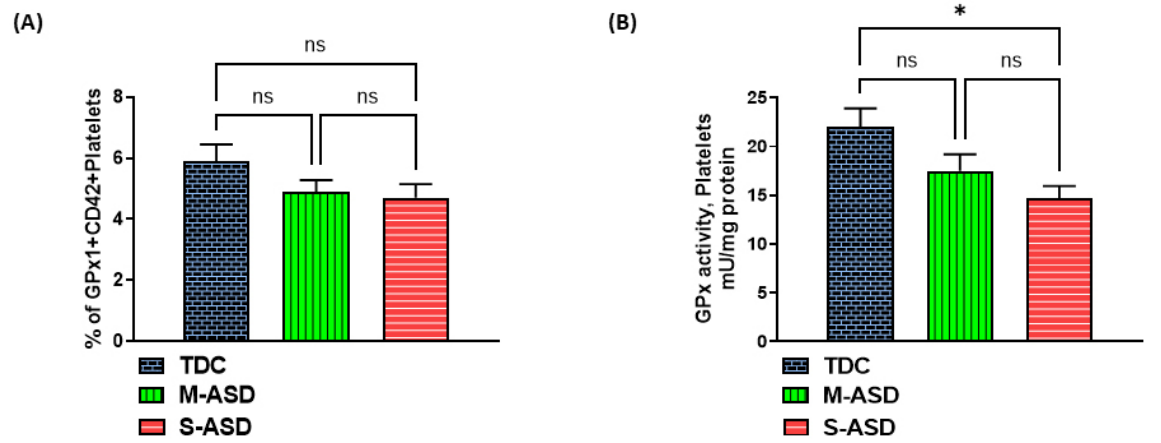

Fig. 2. Expression and activity of glutathione peroxidase (GPx) in platelets of ASD and TDC subjects. (A) % of GPx1+CD42+ cells, and (B) GPx activity in platelets. Data are expressed as mean ± SEM, (TDC group, n = 23; M-ASD group, n = 17; S-ASD group, n = 9). *$p < 0.05$; ns, not significant.

The results showed that the expression of TRxR in platelets was significantly downregulated ($p < 0.01$) in individuals with ASD than that of the TDC group (Fig. 4A,B). It was reflected by a decreased % of double positive immunostaining TRxR1+ andCD42+ cells in ASD group. Moreover, there was a further significant decrease in TrxR1+ expression in platelets with increasing severity as depicted by a significant difference ($p < 0.05$) between M-ASD and S-ASD groups (Fig. 4A,B). Furthermore, there was also a corresponding significant decrease ($p < 0.01$) in TRxR activity in platelets of individuals with ASD, as shown in Fig. 4C. This indicates that the expression as well as activity of TRxR decrease with increasing severity in the platelets of ASD individuals.

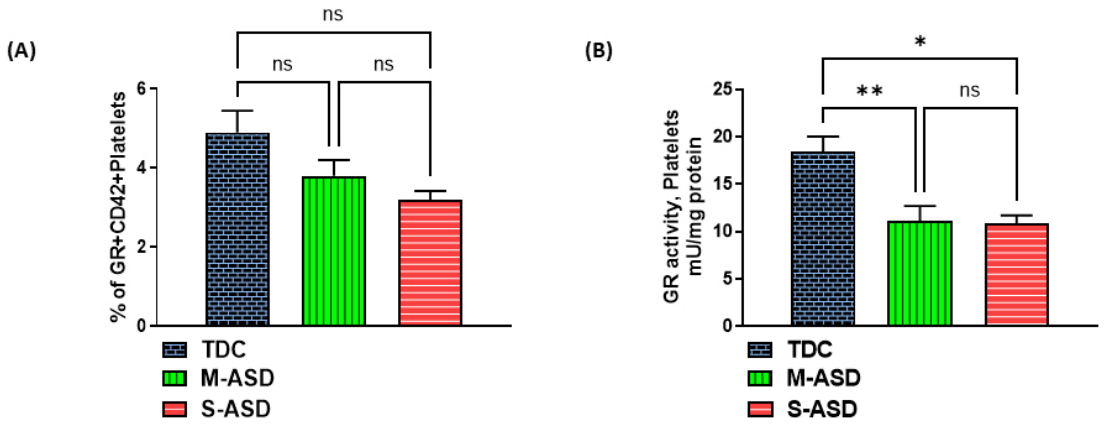

Fig. 3. Expression and activity of glutathione reductase (GR) in platelets of ASD and TDC subjects. (A) % of GR+CD42+ cells, and (B) GR activity in platelets. Data are expressed as mean ± SEM, (TDC group, n = 23; M-ASD group, n = 17; S-ASD group, n = 9). *$p < 0.05$; **$p < 0.01$; ns, not significant.

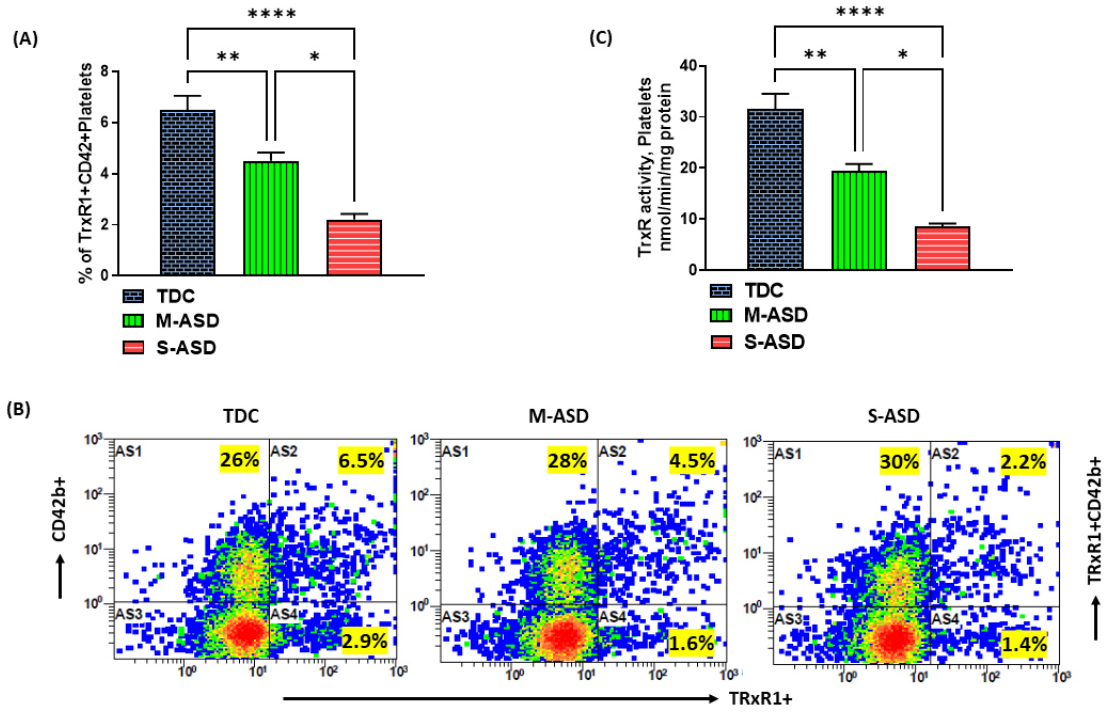

Fig. 4. Expression and activity of thioredoxin reductase (TRxR) in platelets of ASD and TDC subjects. (A) % of TrxR1+CD42+ cells (AS2 quadrant of panel B is represented in this figure), (B) Flow plot showing double positive immunostaining of TrxR1+CD42+ cells, and (C) TrxR activity in platelets. Data are expressed as mean ± SEM, (TDC group, n = 23; M-ASD group, n = 17; S-ASD group, n = 9). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

Elevated Oxidative Markers, iNOS and Nitrotyrosine in Platelets of ASD Individuals

The final aim was to quantify the expression of oxidant enzyme, iNOS and nitrotyrosine which serves as an indicator of protein oxidation and oxidant stress. The observations indicated a significantly higher expression of iNOS ($p < 0.0001$) in platelets of ASD platelets than TDC platelets. It was mirrored by increased % of iNOS+ immunostaining in CD42+ cells in ASD group, as shown in Fig. 5A,B. Further, there was a significantly higher expression of nitrotyrosine ($p < 0.001$) in platelets of ASD platelets than TDC platelets. It was mirrored by increased % of nitrotyosine+ immunostaining in CD42+ cells in ASD group, as shown in Fig. 5C,D. Moreover, there was a further significant increase both in iNOS ($p < 0.0001$) and nitrotyrosine ($p < 0.05$) expression in platelets with increasing severity as depicted by a significant difference between M-ASD and S-ASD groups (Fig. 5A–D). This indicates that there is an increase in iNOS-mediated oxidative stress in ASD platelets

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

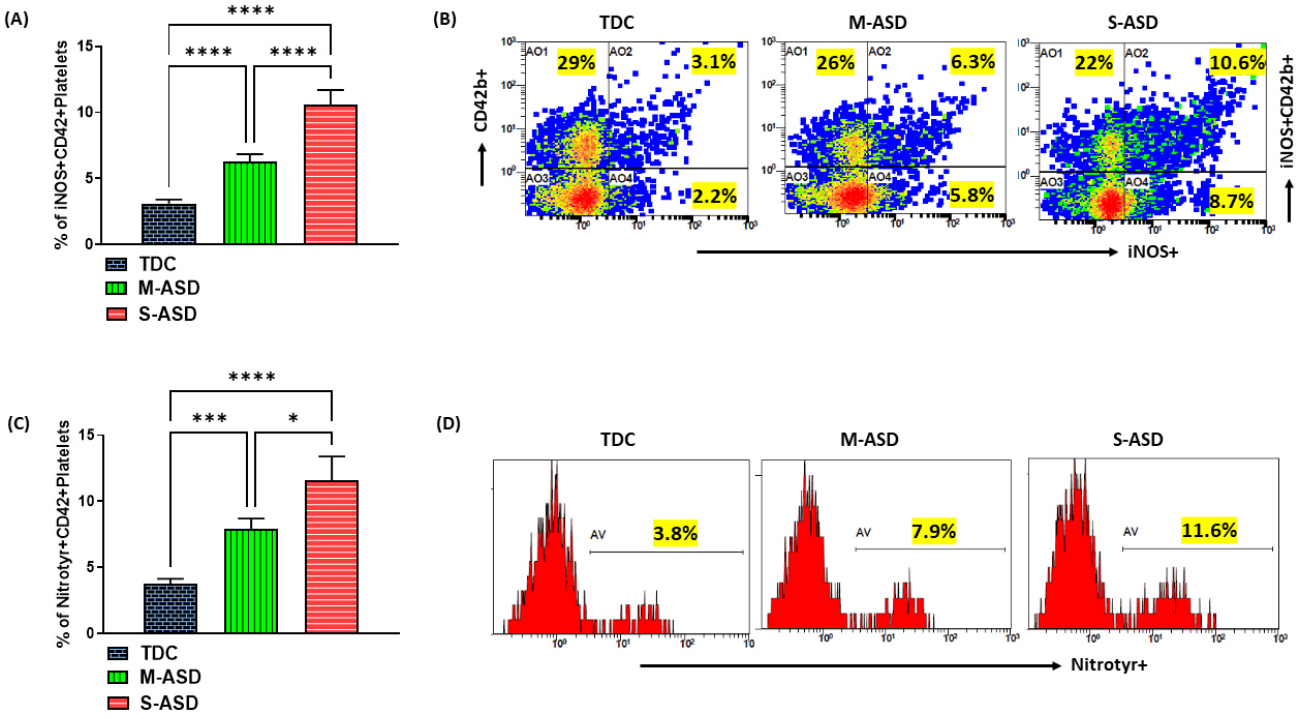

Fig. 5. Expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in platelets of ASD and TDC subjects. (A) % of iNOS+CD42+ cells (AO2 quadrant of panel B is represented in this figure), (B) Flow plot showing double positive immunostaining of iNOS+CD42+ cells, (C) % of Nitrotyrosine+CD42+ cells, and (D) Histogram showing immunostaining of Nitrotyrosine+ cells in platelets (AV represents % of Nitrotyrosine immunostaining in CD42+ platelets). Data are expressed as mean ± SEM, (TDC group, n = 23; M-ASD group, n = 17; S-ASD group, n = 9). *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

which is concomitant with insufficient protection from antioxidants that may lead to oxidative inflammation seen in platelets of individuals with ASD.

Discussion

Individuals with ASD often exhibit disrupted sensory perceptions and long-lasting challenges in social and linguistic communication. The presence of these deficits has a major impact on social behavior and the overall learning experience, both in educational settings and at home [1,4]. These events, which often lead to impaired brain development and immune system function, are believed to be influenced by both genetic and environmental factors. Oxidative stress and antioxidant dysregulation are considered significant contributors to the development of ASD [6,26]. Our work demonstrates dysregulation in enzymatic antioxidants and an increase in iNOS signaling in the peripheral platelets of individuals with ASD for the first time.

Recent studies have provided evidence of oxidative stress in different peripheral immune cells of children with ASD. Various immune cells present in the bloodstream play a crucial role in safeguarding the immune system from both internal and external oxidants, which can arise from pollutants and toxicants. As a result, these immune cells have a sophisticated network of both enzymatic and non-enzymatic antioxidants. When there is a rise in oxidant formation in immune cells, these antioxidants react in a timely way [7,27,28]. This may be understood by considering the interconnected network of enzymatic antioxidants present inside immune cells. Superoxide is eliminated by SOD, which converts it to hydrogen peroxide. The combination of GPx and GR/TRxR activities handles hydrogen peroxide and other lipid peroxides [7,8,27,28]. Previous researches have largely shown lower/higher SOD/GPx/GR activity in plasma/red blood cells (RBCs)/neutrophils/monocytes in individuals with ASD [7,12,18–23]; however, no previous research has investigated enzymatic antioxidants in the peripheral platelets of individuals with ASD, which are probable contributors to inflammation in the periphery and nervous system. In light of this, we planned this investigation to assess the expression and activity of major enzymatic antioxidants (SOD, GPx, TRxR, and GR) in the peripheral platelets of the ASD/TDC groups.

The results of our investigation revealed downregulated levels of SOD protein expression and activity in the platelets of individuals with ASD. SOD is the main enzyme for detoxification of superoxide radicals. As platelet SOD mostly represents the intracellular form, it is conceivable that the inactivation or destruction of intracellular SOD may occur because of excessive oxidant production within

platelets. Previous researches have also shown reduced levels of SOD activity in the serum and RBCs of individuals with ASD [19,21,29].

In the case of platelets, the absence of a nucleus may result in a lack of induction mechanisms due to a lack of transcriptional/translational machinery similar to RBCs. In contrast, the intracellular environment of other immune cells such as neutrophils and monocytes are expected to have greater access to enzymatic antioxidants due to the presence of genetic replenishing mechanisms provided within the nucleus [7,27,28,30].

It seems that the peripheral platelets of individuals with ASD are unable to combat oxidative stress due to the depletion of enzymatic antioxidants. Therefore, rather than reducing the impact of oxidative stress, peripheral platelets exhibit heightened levels of oxidative damage. This is shown by the observed rise in nitrotyrosine production, a recognized indicator of peroxynitrite-induced oxidative damage to proteins, within these cells. Platelets may produce peroxynitrite through iNOS-mediated production of nitric oxide. The presence of a higher percentage of platelets that are positive for both iNOS and nitrotyrosine points towards this possibility in individuals with ASD. Oxidant species such as peroxynitrite cannot be effectively neutralized if enzymatic antioxidants are not functioning properly [27,28].

In a healthy state, primary oxidant species such as nitric oxide are typically generated at minimal levels; however, under inflammatory circumstances, iNOS-derived nitric oxide formation may increase a thousand-fold [17, 31]. iNOS-derived nitric oxide has been shown to play a crucial role in promoting platelet activation. iNOS-deficient platelets showed a significantly inhibited aggregation response, indicating that iNOS plays an important role in platelet activation [32]. The presence of iNOS has also been described in human platelets although at very low levels [32,33]. iNOS expression is reported to be increased during dengue fever infection, leading to increased nitric oxide production according to the severity of the symptoms [33]. iNOS-derived peroxynitrite has also been detected in the platelets of patients with diabetes, which was linked with platelet dysfunction [34]. An increase in iNOS activity may result in the formation of peroxynitrite as observed in our study may inactivate multiple enzymatic targets, leading to dysfunction in cellular function [30,31].

Precise control of the equilibrium between the production of pro-oxidants and their removal by antioxidants is crucial for maintaining the functional stability of platelets. Deficient antioxidant defenses are widely recognized as a significant modulator of aberrant platelet function. Previous studies indicate that the level of oxidants and antioxidants plays a crucial role in determining platelet function [35,36]. SOD is involved in the regulation of platelet activity and the prevention of blood clot formation (thrombosis). It was shown recently that platelet SOD was involved in the regulation of thrombin production, procoagulant platelet response, and artery thrombosis [37]. The decreased activity of SOD and increased formation of peroxynitrite/lipid peroxides are linked to heightened activation responses in platelets [35–37].

Previous researches have shown elevated levels of iNOS in monocytes and neutrophils in individuals with ASD [13,14,38]. Elevated levels of reactive oxidants resulting from impaired mitochondrial function and heightened xanthine oxidase and NADPH oxidase activity have been documented in individuals with ASD [6,16,39]. Oxidative damage to proteins, lipids, and DNA has been observed in the ASD group as reflected by increased levels of chlorotyrosine, lipid peroxides, isoprostanes, and oxo-deoxyguanosine in the peripheral/central nervous system compartment [9–11,20]. Previous studies, including our own and those conducted by others, have shown elevated levels of nitrotyrosine in different immune cells of individuals with ASD [7,14,16,20,38]; however, this investigation has shown the simultaneous presence of enhanced enzymatic oxidative enzyme iNOS and its oxidative end-product nitrotyrosine in the peripheral platelets of individuals with ASD for the first time.

One of the primary antioxidant disulfide reductases responsible for preserving the reduced state of proteins is the TRxR/thioredoxin couple [40]. The ability of thioredoxin to reduce intracellular substrates relies on the activity of TRxR. Thioredoxin not only helps to keep intracellular proteins in their reduced state, but it also safeguards cells from oxidant stress by serving as an electron donor for thioredoxin peroxidases, facilitating the reduction of hydrogen peroxide [40,41]. Recent studies have shown that the TRxR is essential in preventing oxidative stress in T cells, neutrophils, platelets, and endothelium, which are important players in ASD and cardiovascular complications [42–45]. A recent study showed that constitutive expression of TRxR in platelets and its blockade by auranofin resulted in elevated oxidant stress and disturbed the balance of intracellular calcium and procoagulant activity [45]. Therefore, decreased expression/activity of TRxR along with SOD in this study could lead to increased oxidant stress in ASD platelets, which could predispose them to cardiovascular and neuroinflammatory effects.

ASD is often associated with dysregulations in the vasculature, immune system, and central nervous system. Young individuals with ASD are more prone to experiencing cardiovascular diseases (such as heart disease, dyslipidemia, and diabetes), neuroinflammatory/autoimmune disorders, and gastrointestinal troubles (such as increased intestinal permeability, alterations in overall microbiota, abdominal bloating, and gut infection) compared to their non-ASD counterparts [46–48]. Researchers have shown that increased systemic oxidative inflammation can activate vascular endothelial cells and platelets, which could be associated with an altered vascular/neuroinflammatory phe-

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

notype in autism [46,49,50]. Given the significant involvement of platelet activation in these disorders, it is plausible that the observed elevation of oxidative stress in platelets among individuals with ASD may contribute to the development of these associated conditions [49–51].

This study has some limitations. First, this was a cross-sectional study, and participants were not followed over time for confirmation of measured biochemical parameters. To validate the findings of the present investigation, a longitudinal study with a larger sample size may be required. Second, given the recent developments in the field of platelet biology, it would be interesting to investigate the mRNA levels of different oxidative and antioxidative enzymes in platelets, although it is known that platelets do not possess nuclei. Third, this investigation does not evaluate alterations in platelet function, including their capacity for aggregation or participation in thrombus formation. The clinical manifestations associated with ASD may be linked to alterations in platelet function. Therefore, additional research is necessary to address these constraints and better understand platelet function in the context of ASD.

Conclusions

In summary, our investigation demonstrates that the enzymatic antioxidants, SOD and TRxR are decreased, whereas iNOS and nitrotyrosine are increased in peripheral platelets in individuals with ASD. Consequently, ASD platelets experience heightened oxidative stress probably due to an imbalanced antioxidant network. Thus, our research indicates that an imbalanced enzymatic network in peripheral platelets may play a significant role in the development/progression of ASD.

Availability of Data and Materials

This manuscript presents all the data. All the data is available from the corresponding authors upon reasonable request.

Author Contributions

Conceptualization, AN, MMA; Methodology, AN, MMA, SFA, SMA, KA; Software, AN, MMA, SFA, SMA, NOA; Validation, AN, MMA, SFA, SMA, LYA; Formal analysis, AN, MMA, SFA, SMA, NOA, AZA, SAB; Investigation, AN, MMA, SFA, SMA, LYA, KA; Writing—original draft preparation, AN, MMA, SFA; Writing—review and editing, AN, MMA, SFA, SMA, NOA, LYA, KA, SAB, AZA; Supervision, NOA, SAB, LYA. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The local ethical committee at Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia granted approval (IRB Approval number: E-10-220) for this study according to the national guidelines for research on human samples as stated in Declaration of Helsinki. All children who participated in this research had their written permission signed by their parents or legal guardians.

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Conflict of Interest

The authors declare no conflict of interest.

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