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Original article

# Evaluation of extracellular adenine nucleotides hydrolysis in platelets and biomarkers of oxidative stress in Down syndrome individuals

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ABSTRACT

*Purpose:* Down syndrome (DS) is caused by the triplication of chromosome 21. Studies have demonstrated platelets abnormalities and oxidative stress in DS subjects. The enzymes NTPDase, 5′-nucleotidase and adenosine deaminase (ADA) represent an important therapeutic target since they interfere in the extracellular nucleotide pool altering platelet functions. In this study, we evaluated the ectonucleotidases activities and oxidative stress parameters in samples of DS and healthy individuals.
*Methods and results:* The population consisted of 28 subjects with DS and 28 healthy subjects as a control group. Blood was obtained from each subject and used for platelet and serum preparation. NTPDase activity using ATP as substrate was increased in platelets of DS patients in relation to the control group; however, no alterations were observed in the ADP hydrolysis. A decrease in the 5′-nucleotidase activity and an increase in the ADA activity was observed in platelet of DS subjects when compared to healthy individuals ($P < 0.05$). The lipid peroxidation and total thiol content was decreased in serum of DS individuals. Furthermore, superoxide dismutase and catalase activities were increased in whole blood of this group ($P < 0.05$).
*Conclusion:* Alterations in the ectonucleotidase activities in platelets as well as changes in the oxidative stress parameters may contribute to the clinical features of DS.


## 1. Introduction

Down syndrome (DS), the most common genetic disorder, is caused by the triplication of chromosome 21 or part of it [1]. DS occurs in approximately 1 out of 700 live births and is associated with several systemic dysfunctions that include mental retardation and early-onset Alzheimer's disease, obesity, immune disorders and congenital heart defects [2–5].

Data from literature have demonstrated hematologic abnormalities in DS subjects, which include alterations in the platelets, such as changes in their size and in their number [6–8]. Moreover, an increase in the platelet membrane fluidity, deficiencies in the mitochondrial enzymes, a decrease in the glutamate uptake and calcium content, and a decrease in the ATPase activity were also described in platelets of DS individuals [9–13]. The clinical significance of these platelet alterations is not yet well understood but it may be associated with the cerebral venous thrombosis incidence in DS subjects [14,15].

Platelets constitute the first line of defense when normal vessels are injured thus they have a critical role in hemostasis and thrombosis mechanisms [16]. Platelet adhesion, aggregation and further platelet recruitment culminate in hemostatic plug formation at the site of vascular injury [17,18]. It is well established that adenine nucleotides and nucleosides such as ATP, ADP and adenosine modulate the platelet aggregation [19]. ADP is a well known agonist involved in the recruitment and aggregation of platelets at sites of vascular injury by activating purinergic receptors on the platelet membrane [20]. Furthermore, adenosine

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is a potent inhibitor of platelet aggregation and acting as a cardioprotector molecule [21,22].

The effects of ATP, ADP and adenosine in the platelet function are modulated by ectoenzymes such as NTPDase, 5′-nucleotidase and adenosine deaminase (ADA) present in the platelet membrane [23]. These enzymes constitute an organized enzymatic chain that begins with the action of E-NTPDase, which catalyzes the hydrolysis of ATP and ADP to AMP [23]. The AMP is subsequently hydrolyzed by 5′-nucleotidase to adenosine, which is degraded by ADA to inosine [24,25]. Together, these enzymes represent an important therapeutic target since they interfere in the extracellular pool nucleotides and nucleosides [26]. In fact, a growing number of evidences have indicated that the activity and expression of ectonucleotidases in platelets is deregulated in several human diseases [27–29].

It has been also demonstrated that reactive oxidant species (ROS) can alter the platelet function and that ectonucleotidase activities are altered in platelets in pathologies associated with oxidative stress [27–30]. In this line, there are studies reporting that oxidative stress plays a key role in the pathogenesis of DS. The oxidative stress in DS may result in the over expression of enzyme Cu/Zn superoxide dismutase (SOD1) encoded by genes on chromosome 21 and by abnormalities of mitochondrial functions [31,32]. Although oxidative stress parameters have been investigated in a number of studies, the role of these alterations remained controversial in DS.

In this line, due the fact that NTPDase, 5′-nucleotidase as well ADA controls the extracellular levels of important molecules involved in the platelets function and considering that the mechanisms in relation the hematological changes of DS are not yet well understood the aim of this study was to evaluate the ectonucleotidases activities in platelets of the DS subjects. In addition, considering that ectonucleotidases are membrane enzymes possible alterations in these proteins could be mediated by oxidative stress mechanisms. In this sense, the markers of oxidative stress in blood of DS were also evaluated in order to cooperate for the better understanding the platelets dysfunctions associated with DS.

## 2. Materials and methods

### 2.1. Subjects

The study was performed in 28 individuals with DS and 28 healthy controls matched by age and sex from Pelotas, RS, Brazil. The general characteristics of the participants are shown in Table 1. Each participant or a legal guardian signed an informed consent to participate in this study and the Human Ethics Committee from the Federal University of Pelotas approved the study protocol.

### 2.2. Sample collection and preparation

Ten milliliters of blood was obtained from each individual with DS and used for sample preparation. The same procedure was carried out for the control group. Whole blood was centrifuged at $1000 \times g$ for 10 min and serum was removed by aspiration, aliquoted and frozen at −80 °C until biochemical determinations.

Platelet-rich plasma (PRP) was prepared by the method of Pilla et al. [33] Total blood was collected with sodium citrate as anticoagulant and centrifuged at $160 \times g$ for 10 min. After this, PRP was centrifuged at $1400 \times g$ for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0. Platelet pellets were resuspended in HEPES buffer and used to determine enzymatic activities.

**Table 1**
Characteristics of Down syndrome (DS) and control individuals. Variables such as age are presented as mean ± SEM.

| | Control | DS |
|---|---|---|
| Number | 28 | 28 |
| Women | 15 | 12 |
| Men | 13 | 16 |
| Age (women) | 24.12 ± 5.54 | 26.20 ± 5.76 |
| Age (men) | 23.67 ± 3.98 | 28.88 ± 6.94 |

### 2.3. NTPDase and 5′-nucleotidase assays

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 μL, as described by Pilla et al. [33]. For AMP hydrolysis, the medium reaction was used as previously described except that 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$.

Twenty microliters of the enzyme preparation (8–12 μg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1 mM, and AMP at a final concentration of 2 mM. The incubation time was 60 min. Both enzyme assays were stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. [34] using malachite green as the colorimetric reagent and $KH_2PO_4$ as standard. Control samples were carried out to correct for non-enzymatic hydrolysis of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein.

### 2.4. Adenosine deaminase determination

Adenosine deaminase (ADA) from platelets was determined according to Giusti and Galanti [35]. Briefly, 50 μL of platelets reacted with 21 mmol/L of adenosine, pH 6.5, incubated at 37 °C for 60 min. This method is based on the direct production of ammonia when ADA acts in excess of adenosine. Results were expressed in units per mg of protein (U/mg of protein). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

### 2.5. Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring TBARS in serum at 532 nm according to a modified method of Ohkawa et al. [36]. Results were expressed as nmol TBARS/mg of protein.

### 2.6. Total sulfhydryl content

This assay was performed in serum as described by Aksenov and Markesbery [37], which is based on the reduction of DTNB by thiols and in turn it becomes oxidized (disulfide) generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. The reaction was started by the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Results were reported as nmol TNB/mg protein.

### 2.7. Catalase (CAT) and superoxide dismutase (SOD) activities in whole blood

The determination of CAT activity was carried out in accordance with the modified method by Nelson and Kiesow [38]. This assay involves changes in the absorbance at 240 nm, for 2 min, due to