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Review

Morphological changes in erythrocytes of people with type 2 diabetes mellitus evaluated with atomic force microscopy: A brief review

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ABSTRACT

Prevalence of type 2 diabetes mellitus (T2DM) has been increasing worldwide. Cardiovascular diseases are one of the main causes of death among people with T2DM. Morphological changes in erythrocytes have been associated with higher risk of cardiovascular diseases. Atomic force microscopy (AFM) is a new technique that allows non-invasive imaging of cells and the evaluation of changes in mechanical properties.

Aim: To evaluate by AFM the erythrocytes morphological changes of people with T2DM

Methods: Search was conducted from in PubMed, ScienceDirect, Scielo, and Lilacs. Erythrocyte, type 2 Diabetes Mellitus and, Microscopy, Atomic Force were the keywords used for the search. Papers included were cross-sectional studies performed in humans.

Results: Five of seven articles fulfilled the inclusion criteria. Compared with healthy cells, the erythrocytes from individuals affected by T2DM had morphological changes such as a decreased concave depth, diameter, height and a deformation index, while axial ratio, stiffness, adhesive force, aggregation, and rigidity index were increased. The results regarding the erythrocyte roughness were inconclusive.

Conclusions: The AFM is an excellent instrument to study the altered erythrocytes of subjects affected by T2DM. Morphology changes in erythrocytes could lead to cardiovascular events, which are major complications in people living with this disease

1. Introduction

Diabetes is one of the largest health emergencies of the 21st century. Type 2 diabetes mellitus (T2DM) is the most common condition, and its prevalence has been increasing worldwide in the last few decades. Moreover, it is estimated by 2030 there will be 552 millions of people affected by this disease. Besides, cardiovascular disease is one of the leading causes of death among subjects affected by T2DM and could account for 50% or more of the deaths (International Diabetes Federation, 2015).

Morphology changes in the structure, shape, and function of the erythrocytes, as well as some rheological changes such as decreased deformability and increased aggregation have been associated with higher risk of cardiovascular diseases (Diez-Silva et al., 2010; Soma and Pretorius, 2015). Erythrocytes are biconcave cells, without a nucleus or mitochondria and take part in the inflammatory response. Its membrane comprises two domains, an overlaying asymmetric phospholipid bilayer membrane and an underlying spectrin-actin cytoskeletal interconnected by junctional complexes (Almqvist et al., 1994; Liu et al., 2003). Some of these abnormalities in individuals with diabetes are caused by the reduction in the cholesterol to phospholipid ratio in the lipid bilayer of the erythrocyte membrane (Soma and Pretorius, 2015). Also, there is an enhanced fibrinogen production by insulin, which contributes to hyperfibrinogenemia and therefore to an increased cardiovascular risk through aggregation of erythrocytes (Barazzoni et al., 2003).

Abbreviations: T2DM, type 2 diabetes mellitus; AFM, atomic force microscopy; STROBE, strengthening the reporting of observational studies in epidemiology; SEM, scanning electron microscopy.

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Instruments such as the atomic force microscopy (AFM) are ideal for measuring alterations in the change of shape, roughness, adhesive force (a proxy of aggregation), and rigidity (a proxy of deformability). The AFM is a new technique that allows non-invasive imaging of cells to evaluate changes in mechanical properties of single cells at micrometer or nanometer resolution with minimal sample preparation (Santos and Castanho, 2004). Three modes of operation are available to measure the different characteristics of the erythrocytes: 1. Contact (tip remains in direct contact with the sample when translated over the surface); 2. Tapping mode (image soft, fragile, and adhesive surfaces without damage to evaluate the erythrocytes morphology), and 3. Force spectroscopy (evaluates the strength of the interaction between the tip and the sample such as the adhesive force and stiffness) (Carvalho and Santos, 2012; Russell et al., 2001).

A recent review described several AFM applications to analyze human erythrocytes (Mukherjee et al., 2015). However, to our knowledge, no reviews are evaluating specific differences between erythrocytes of healthy individuals compared to those affected by T2DM. The aim of this paper was to examine the literature related to the morphological changes occurring in the erythrocytes of individuals affected by T2DM compared to healthy people by AFM.

2. Materials and methods

The search was conducted from November 2016 to June 2017 in databases such as PubMed, ScienceDirect, Scielo, and Lilacs. The search was performed with the following keywords: "erythrocytes," "morphology," "type 2 Diabetes Mellitus" and "Microscopy; Atomic Force" (previous validation in MESH). The outcomes of interest were the morphological changes in the erythrocytes of healthy individuals and people living with T2DM.

The papers included in this review were cross-sectional studies performed in humans, written in English or Spanish, and there was not a range of years considered since few studies compared erythrocytes of healthy people and subjects affected by T2DM. The exclusion criteria were being a review, book chapter, letters to the editor, not having a control group and full text not available. Once the papers fulfilled the inclusion criteria, they were evaluated by the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist (Berra et al., 2008; Vandenbroucke et al., 2009).

The data such as objectives, sample, method, and results were extracted from each article. The obtained results are reported as means, standard deviations or frequencies.

3. Results

The initial search yielded 27 articles; however, only seven of them described erythrocytes changes in people affected by T2DM or used the AFM as the instrument to quantify cell alterations. Among the latter sample, five articles fulfilled the inclusion criteria (Fig. 1). Once the studies were included, they were assessed by the STROBE evaluation, which identified that 40% of them had a high study quality and the rest a medium quality. Table 1 shows the results of the STROBE evaluation and the objectives of the studies.

3.1. Sample size and study groups

The sample size of the studies was relatively small; almost all of the studies (80%) had less than 16 individuals per group. Also, to compare the erythrocytes of healthy individuals and people living with T2DM studies include two or three study groups. Healthy, non-smoking subjects formed the control group while subjects affected by T2DM (in some studies from diabetic clinics) formed the comparison group. Only two studies (Jin et al., 2010; Starodubtseva et al., 2008) had three groups: one with individuals affected by T2DM, and two groups of healthy individuals (younger adults and the other).

3.2. Sample preparation and analysis

In Table 2 are described the characteristics of the sample preparation and the AFM measurement. Studies reported multiple ways of preparing the erythrocytes for its analysis, which included dilution with Phosphate Buffer Saline (40% of the studies), and fixation/suspended with glutaraldehyde at different concentrations (40%) and the

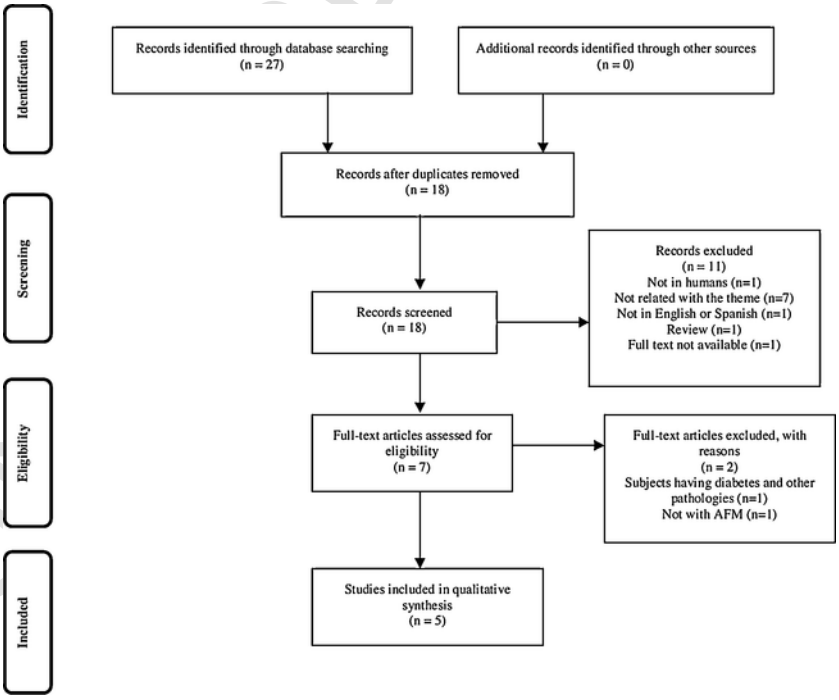

Fig. 1. Flowchart of the review screening process.

Table 1
Results of the STROBE evaluation and the objectives of the included studies.

Author	Year	Country	Objective	Study quality STROBE
Ciasca et al. (2015)	2015	Italy	Measure the stiffness of normal and pathological RBC by AFM under physiological conditions	Medium
Pretorius et al. (2015)	2015	South Africa	Study the clot structure and RBC structure of patients with T2DM	High
Buys et al. (2013)	2013	South Africa	Investigate the membrane roughness and changes in diabetes using AFM and correlated with SEM. Compare the results from both techniques with RBCs of healthy individuals	Medium
Jin et al. (2010)	2010	China	Detect the changes in morphological and biomechanical properties of RBCs from the healthy people and type 2 diabetes patients	High
Starodubtseva et al. (2008)	2008	Belarus	Study structural and mechanical state of RBC in patients with T2DM by AFM	Medium

T2DM: type 2 diabetes mellitus; AFM = atomic force microscopy; SEM: scanning electron microscopy; RBC: red blood cells.

less common fixation with formaldehyde (one study). On the other hand, most of the studies evaluated five to ten erythrocytes from each subject.

Although AFM measures obtained all morphological and mechanical characteristics of the erythrocytes, some studies complemented the information with other instruments such as light microscopy, scanning electron microscopy (SEM) and a viscometer. The contact mode was the most frequent mode employed for AFM evaluations. On the other hand, not all the studies reported the environment (air or liquid) in which the measure was performed, only one study reported doing the measure in a liquid environment (Ciasca et al., 2015).

3.3. Erythrocytes morphology changes

The morphology changes evaluated in the erythrocytes were diameter, height, concave depth and axial ratio; and the mechanical properties were roughness, adhesive force, and stiffness by Youngs modulus. Also, some studies evaluated hemorheology properties such as deformability, aggregation, and rigidity. In Table 3 are shown the studies results.

This review found that compared with healthy cells, the erythrocytes from individuals affected by T2DM have a decreased concave depth, diameter, height and a deformation index, whereas axial ratio, stiffness, adhesive force, aggregation, and rigidity index were increased. Moreover, although the three studies that evaluated stiffness found an increased value in people affected by T2DM, they report the values in different units. Table 4 shows the normal values of erythrocytes characteristics reported in the studies included in this review, and the values obtained by our research group. Besides, our research team obtained AFM and SEM images of the typical erythrocyte morphology of healthy individuals showed in Figs. 2 and 3.

4. Discussion

This review found that erythrocytes of individuals with T2DM evaluated by AFM display changes in the morphological, mechanical, and hemorheological properties compared to healthy cells. AFM permits a high-resolution imaging, simple sample preparation (without damage), the capability to measure in a liquid environment, and the ability to obtain information regarding cell roughness, adhesive force, and rigidity (De Oliveira et al., 2012; Mukherjee et al., 2014). Also, SEM is used to compare the changes in morphology and the in the membrane of erythrocytes of people affected by T2DM compared with healthy cells. The advantages of SEM is the large depth of field (three-dimensional appearance) and the ability to obtain compositional information using characteristic x-rays (Goldsetin et al., 2003). In fact, a group of researchers evaluated by SEM and AFM the effect of an antioxidant (which could have a positive impact on deformability and protein-membrane structure) in the membrane of T2DM erythrocytes. They found a smoother membrane surface, which could indicate a change in eryptotic nature of erythrocytes (Visser et al., 2017). Therefore, the combined use of both microscopes offers new opportunities for nanoscale imaging to visualize changes in morphology, deformability, membrane elasticity, roughness and stiffness. The information provided by both AFM and SEM could be used for better diagnosis focused on precision medicine to individualize treatments and evaluate treatment adherence to different inflammatory diseases (Pretorius et al., 2016b).

Most of the studies in this review only evaluated five to eight cells of each subject. The reason is that it has been reported that scanning a small number of erythrocytes is a reliable measure to assess roughness (Girasole et al., 2010). Another reason to evaluate few erythrocytes from each subject from a small sample size is that AFM evaluation of a single cell is time-consuming; sometimes a single image can take an hour or more. However, this depends on the AFM used, the time spend by line, how many lines do you want in the image, and how long it takes to find a suitable cell.

Also, some studies had a group with healthy adults from 50 to 65 years old; it has been reported that the aging process itself increases morphological changes in erythrocytes (Pandey and Rizvi, 2010). For example, a study observed that although the presence of cardiovascular risk influences rheological properties (blood viscosity, plasma viscosity, erythrocyte aggregation and erythrocyte deformability), aging itself is associated with deterioration of rheological blood behavior, mostly related to inflammatory and lipidic changes (Vayá et al., 2013).

On the other hand, alterations in the structure, shape, and function occur in the erythrocytes as a consequence of diabetes mellitus. For example, we found a decrease in the cell diameter, an increase in the axial ratio, as well as poikilocytosis (abnormal variation in the erythrocyte shape) and anisocytosis (abnormal variation in the erythrocyte size). In fact, a study reported the shape of erythrocytes from 30 individuals with T2DM by using a light microscope, found an average in the diameter of 6.83 μm ± 0.66 (Neamtu et al., 2015), which agrees

Table 2
Summary of studies characterizing morphological and biomechanical changes in erythrocytes of people living with T2DM.

Author	Study groups	Sample preparation	AFM measurement
Ciasca et al. (2015)	**Control group**: n = 15, healthy **Diabetic patients**: n = 5, Patients with T2DM	Suspended in 10 mM of PBS and deposited on a poly-L-lysine coated petri dish.	Mode: N/I Cells evaluated: Ten Instrument: JPK Nanowizard II (Berlin, Germany) Spectroscopy: Yes
Pretorius et al. (2015)	**Control group:** n = 25, healthy, non smoking, without chronic conditions **Diabetic patient**: n = 69, randomly chosen from a diabetic clinic	RBCs were fixed in formaldehyde followed by a dehydration and drop in a glass cover slip with hexamethyldisilazane.	Mode: N/I Cells evaluated: Ten Instrument: Dimension Icon with ScanAsyst, Bruker, USA Spectroscopy: Yes
Jin et al. (2010)	**Young healthy people (YHP):** n = 8, age 25–27, healthy **Healthy old people (OHP): n = 4, age 55–65, healthy** **Type 2 diabetes patients (ODP)**: n = 5, age 55–65, with dx of T2DM	RBC were diluted in PBS and centrifugalized (3000 rpm, 10 min). Then, the suspension was dropped on glass cover slips and dried in air.	Mode: contact Cells evaluated: Five Instrument: Autoprobe CP Research, Veeco, USA Spectroscopy: Yes
Buys et al. (2013)	**Control group:** n = 10, healthy individuals, non-smoking and without medication, males and females **Diabetic patients**: n = 10, dx of T2DM but clinically stable, from a diabetic clinic, males and females	RBC were suspended in glutaraldehyde, rinsed with PBS and post-fixed with OsO4. Then dehydrated with ethanol series and dried on a glass coverslip using hexametildisilizane.	Mode: tapping Cells evaluated: Eight Instrument: Dimension Icon, Bruker, USA Spectroscopy: No
Starodubtseva et al. (2008)	**G1**: n = 15, patients with T2DM of a Regional Clinical Endocrinology Center, age over 50 **G2**: n = 6, healthy volunteers, age over 50 **G3**: n = 3, healthy volunteers, age under 25	RBC were fixed in 1% glutaraldehyde and washed once in a buffer and twice in distilled water, placed onto slides and dried.	Mode: contact Cells evaluated: Eight Instrument: NT 206 AFM (MicroTestMAchine) Spectroscopy: No

T2DM: type 2 diabetes mellitus; RBCs: red blood cells; PBS: phosphate buffer saline; AFM: atomic force microscopy: N/I: no information.

with the reported by Buys et al. Buys et al. (2013). However, they did not found a significant statistical difference compared to the control group. Also, they found that 46.6% of individuals of the study group presented anisocytosis and 30.3% poikilocytosis compared to 3% in the control group, which agrees with the reported by Starodubtseva et al. (Starodubtseva et al., 2008).

Another cause of morphology alteration in people with T2DM is eryptosis, which produces cell shrinkage and membrane scrambling due to the exposure of erythrocytes to excessive glucose concentration. Before senescence, erythrocytes may experience injury and disruption, which may compromise their integrity and survival. Under this condition, the affected erythrocyte may undergo suicidal death or eryptosis to eliminate defective erythrocytes before hemolysis. However, since erythrocytes lack nuclei and mitochondria, they require several critical apoptotic events, such as mitochondrial depolarization and condensation of nuclear chromatin. Therefore, the mechanism is stimulated by an increase in cytosolic Ca^{2+} (activated by a scramblase). Then, the phosphatidylserine flips (due to flippase inhibition, activation of the scramblase and the involvement of a floppase) leading to expose it at the erythrocyte surface producing membrane asymmetry. Finally, this fosters the adherence of eryptotic cells to endothelial cells and macrophages with subsequent engulfment and intracellular degradation (Lang et al., 2012; Pretorius et al., 2016a). This enhanced eryptosis has been reported in chronic degenerative diseases (Lang et al., 2012). So, it is possible that membrane changes in erythrocytes could be useful as a biological indicator to determine early stages of some diseases such as diabetes mellitus and renal insufficiency (Chang et al., 2017; Ertan et al., 2017).

Cell roughness is a morphological parameter that links the development of nanoscale changes on the cell membrane, mainly associated with the occurrence of membrane-skeleton defects independent from the overall cell shape (Girasole et al., 2012). Regarding the results of the erythrocyte roughness, the findings were inconclusive since one study mentions an increase in people with T2DM compared to healthy (Jin et al., 2010), while another study states the opposite (Buys et al., 2013). However, it has been reported that roughness of ill erythrocytes can be 50% smaller compared to healthy cells. The roughness of the cell membrane correlates well with the functional status of the cell since it is an indicator of the skeletal integrity of the membrane and the cells health (Antonio et al., 2012).

Furthermore, most studies carried out the quantitative evaluation of the membrane stiffness using the Youngs Modulus determination. An increased stiffness indicates a decreased deformability, which is common in inflammatory diseases such as diabetes, coronary diseases, and hypertension. For example, a study found that stiffness from diabetic patients might exceed the mean value obtained for normal erythrocytes

Table 3
Erythrocytes morphology changes reported in the studies.

Author	Results		
Ciasca et al. (2015)		**Stiffness (kPa)**[a] Control group: 1.82 ± 0.20 T2DM group: 2.52 ± 0.58	
Pretorius et al. (2015)	**Axial ratio**[b] Control group: 1.14 ± 0.15 T2DM group: 1.25 ± 0.27 ($p < 0.001$)	**Stiffness (MPa)**[a] Control group: 46,710 ± 39,210 T2DM group: 56,483 ± 64,418 ($p < 0.001$)	
Jin et al. (2010)	**Concave depth (nm)**[a] YHP: 1607.2 ± 226.4 OHP: 1202.2 ± 131.7 ODP: 1076.1 ± 117.9 **Adhesive force (pN)**[a] YHP: 200 ± 38 OHP: 420 ± 25 ODP: 510 ± 63	**Stiffness (x10^5 N/m^2)**[a] YHP: 1.04 ± 0.19 OHP: 1.53 ± 0.41 ODP: 1.78 ± 0.39 **Average roughness (nm)**[a] YHP: 586.3 ± 52.4 OHP: 690.2 ± 71.7 ODP: 1333.5 ± 55.2	**Deformation index**[b] YHP: 0.76 OHP: 0.71 ODP: 0.55 **Aggregation index**[b] YHP: 2.15 OHP: 2.50 ODP: 3.42 **Rigidity index**[b] YHP: 4.05 OHP: 4.26 ODP: 4.51
Buys et al. (2013)	**Concave depth (nm)**[a] Control: 358.2 ± 117.6 Diabetic: 153.9 ± 0.58.6 ($p < 0.01$) **Thickness (μm)**[a] Control: 2.68 ± 0.04 Diabetic: 1.58 ± 0.11 ($p < 0.001$)	**Roughness (nm): 1st order**[a] Control: 3.099 ± 0.499 Diabetic: 1.70 ± 0.13 ($p < 0.01$) **Diameter (μm)**[a] Control: 7.22 ± 0.16 Diabetic: 6.80 ± 0.08 ($p < 0.05$)	**2nd order (nm)**[a] Control: 4.12 ± 0.51 Diabetic: 1.66 ± 6.42 ($p < 0.01$) **3rd order (nm)**[a] Control: 1.72 ± 0.199 Diabetic: 0.82 ± 0.11 ($p < 0.01$)
Starodubtseva et al. (2008)	**Normocytes**[a] Healthy people: 62% People with T2DM: 72%	**Poikilocytosis** and **anisocytosis** was characteristic of patients with T2DM.	**Fractal dimension**[a] G1: 2.859 ± 0.089 G2: 2.848 ± 0.107 G3: 2.837 ± 0.116 ($p < 0.05$)

AFM: atomic force microscopy; T2DM: type 2 diabetes mellitus; YHP: young healthy people; OHP: old healthy people; ODP: old diabetic patients.
[a] Measured with AFM.
[b] Measured with other instruments.

Table 4
Normal erythrocytes characteristics and their comparison with the studies information.

Erythrocyte characteristic	Normal value	Research group data*	Studies in the review
Diameter	7–8.7 μm (Price-Jones, 1929)	7.6 ± 0.65	In accordance (Buys et al., 2013)
Axial ratio[a]	1 μm (Pretorius et al., 2015)	1.08 ± 0.11	In accordance (Pretorius et al., 2015)
Thickness	1.5–2.5 μm (Chen et al., 2002)	N/I	In accordance (Buys et al., 2013)
Thickness[b]	0.6 μm□(Dulińska et al., 2006)	0.53 ± 0.3	N/I
Roughness	3.18 ± 0.22 nm (Girasole et al., 2010)	3.3 ± 1.3	In accordance (Buys et al., 2013)
Stiffness	N/I	N/I	The studies reported different values (Ciasca et al., 2015; Jin et al., 2010; Pretorius et al., 2015)

N/I = no information.
* Values were obtained from five erythrocytes of each healthy individual. Sample information: individuals from San Luis Potosi, Mexico, mean age of 20.3 ± 6.8 and without reported diseases.
[a] A value of 1 represents a perfect circle.
[b] Erythrocytes on dried blood films.

by a factor of four. This altered stiffness could be due to alterations in the chemical composition of the erythrocyte membrane, especially in the ratio between phospholipids and cholesterol content (Lekka et al., 2005).

Another modification occurring in erythrocytes is rigidity. The significant increase in the index of rigidity in diabetes compared to healthy controls is attributed to the glycosylation of membrane proteins, which is primarily responsible for the significant decrease in erythrocyte deformability (Symeonidis et al., 2001). In fact, a study observed that erythrocytes from diabetic patients were more rigid and had reduced deformability compared to healthy participants, this is due to the interaction of hemoglobin with the membrane that contributes to the cellular rigidity and alteration of the membrane (Desouky, 2016). Deformability is the ability of the cell to deform under applied shear stress, which is necessary to pass through large and small vessels of the cardiovascular system. In individuals with T2DM, the deformability of the erythrocyte membrane is decreased due to lipid peroxidation and because proteins are heavily glycosylated (especially on beta-spectrin, ankyrin and protein 4.2) (Shin et al., 2007). This impaired deformability can increase blood viscosity leading to an increase in shear stress on the endothelial wall, which can result −in turn– in thrombotic events (Singh and Shin, 2009; Soma and Pretorius, 2015).

Likewise, aggregation is modified by the composition of erythrocyte membrane and plasma proteins (fibrinogen and globulin); when fibrinogen levels are increased, aggregation is enhanced (Soma and Pretorius, 2015). This altered aggregation is responsible for the increased viscosity at low shear rates in blood. In diabetic patients with-

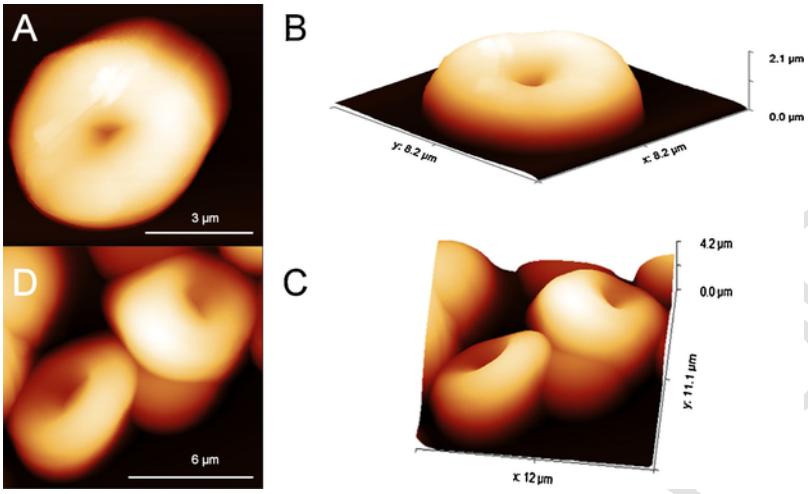

Fig. 2. Atomic force microscope images of typical erythrocyte morphology.Erythrocytes from healthy individuals scanned with atomic force microscopy (Nanosurf Flex C3000 Controller, Switzerland) in air environment and using tapping mode at the Autonomous University of San Luis Potosi, Mexico. A and D present a 2D view, B and C present a 3D view.

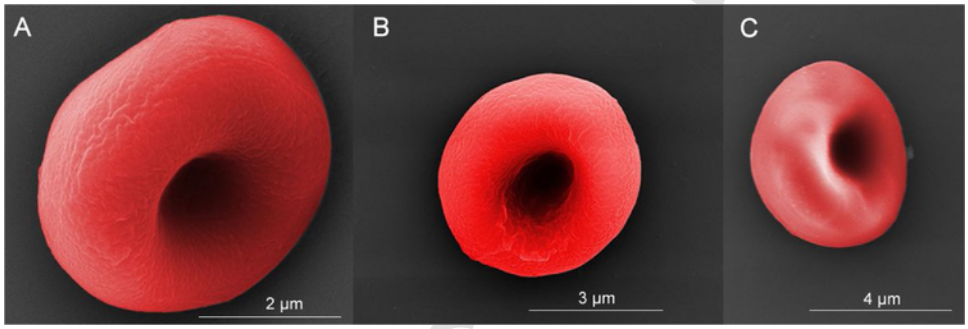

Fig. 3. Scanning electron microscope images of typical erythrocyte morphology.Erythrocytes from healthy individuals obtained with a scanning electron microscope (Inspect S50 FEI Company, Eindhoven, Netherlands). Terahertz Science and Technology National Lab (LANCYTT), Mexico. A, B and C are erythrocytes from healthy individuals showing the typical morphology.

out micro and macroangiopathy the hemorheological disturbances are associated with erythrocyte hyperaggregation (Singh and Shin, 2009).

This review was focused on the morphological changes in erythrocytes of subjects affected by T2DM. However, it was difficult to compare the studies due to different variables and instruments were used. The main strength of the review was that the selected studies described morphological traits of erythrocytes evaluated by AFM. Besides, the studies were assessed using the STROBE list so that only high, and medium quality studies were included.

5. Conclusions

The AFM is an excellent instrument to study topography and mechanical properties of living and fixed cells at a nanoscale. AFM allowed to observed early changes in the erythrocytes morphology of people living with T2DM compared with healthy population. Furthermore, due to erythrocytes are influenced very easily by pro-inflammatory molecules and oxidative stress they are a good health indicator and could provide valuable information regarding the disease status. For example, changes in erythrocytes morphology could predict cardiovascular events, which are significant complications for people living with T2DM. For these reasons, erythrocytes evaluation with the AFM might be routinely used as a more accurate early diagnostic for T2DM and cardiovascular diseases. Besides, in a few years, physicians could evaluate the erythrocytes to provide an individualized treatment and to assess its treatment adherence. However, more studies are needed to elucidate the direction of the change in the erythrocyte membrane roughness, to determine the normal stiffness value and to evaluate other populations to assess if they present similar results. For example, countries from Latin America could benefit from research related to changes in erythrocytes in diabetes population since they have a substantial prevalence of T2DM.

Conflicts of interest

None.

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