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Subcellular Disorders Associated with Autism Peripheral Blood

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ABSTRACT

Autism is a pervasive neurodevelopmental disorder that appears in the first 3 years of life. Causes of autism have not yet been recognized and for unknown reason the brain's normal development of social and communication skills is affected. In view of the fact that some individuals might show only slight disturbances in behavior or mild developmental delays, professional diagnosis is difficult. Since, blood is a major factor in the welfare of individuals, the objective of the study is to assess features of blood of autistic children. Blood samples were obtained from the fingers of autistic children and their mothers. For light microscopy, blood specimens smeared thinly on glass slides, dried, fixed and stained with Leishman's. For electron microscopic examination blood buffy coat was fixed with $_4F_1G$ and ultrathin sections were double stained with uranyl acetate and Lead citrate. Light micrographs of autistic child's peripheral blood showed remarkable erythrocytes morphological alterations. Blood of autistic mothers showed alterations analogous to those observed in their children but less frequent. Ultra structurally, nuclear changes including, nuclear pocket type I, membrane blebbing as well as, absence of mitochondria was noticed in most leukocytes. Platelets alterations were also detected. It could be concluded that autism is associated with comparable alterations in blood cells in autistic candidates and their mothers. The erythrocyte alterations collectively, indicated decreased oxygen capacity of erythrocytes in blood circulation including, circulation in epitomes of fetal brain. Sub cellular alterations in leukocytes especially lymphocytes and plasma cells resulted in their malfunctions.

Key words: Autism, blood, ultrastructure, mitochondria, nuclear abnormalities

INTRODUCTION

Over the last years there has been an explosion of interest in autism which is a brain developmental disorder diagnosed in early infancy (Dover and Couteur, 2007). A period of normal development may be all of a sudden followed by failure of acquired skills and delay in the gaining of new ones (Yeargin-Allsopp *et al*., 2003). It may or may not be accompanied by mental retardation.

Widespread abnormalities characterize autism including, communication difficulties, inability to fit into the society, inappropriate repetitive stereotypical behavior, language difficulty (Rapin and Tuchman, 2008), defects in awareness, cognition, learning and sensory functioning (Forborne *et al*., 2006; Newschaffer *et al*., 2007). Rapin and Tuchman (2008) added severely limited

interests and symptoms that do not involve sickness, fragility, or emotional disturbance. The severity of symptoms can range from almost hardly noticeable to profoundly disabling and several other "autistic-like" conditions that, together with autism, are referred to as Autism Spectrum Disorders (ASD) (London, 2000).

Despite the extensive studies upon the potential factors that have been proposed, causes of autism remain elusive. Considerable research is underway worldwide to spotlight on the primary and secondary factors associated with autism. There is a considerable evidence that the primary factors contribute to the symptoms of autism is determined largely by genetics. Most investigators believe that certain genes a child inherits from their parents could make them more vulnerable to developing an ASD (Gupta and State, 2006).

Yang and Gill (2007) believed that one of the most consistent abnormalities in autism is the decreased cerebellar Purkinje neurons linked to genetic factors and reporting that the genes involved in the growth and the differentiation of the Purkinje cells would be essential candidate genes for autism. Some investigators have argued that autism is not primarily caused by genes but also by environmental factors. The theory is that anyone is born with a resistance to an ASD but the ASD develops only if that person is exposed to a specific environmental trigger (secondary factors) responsible, or partially responsible, for the symptoms of the ASD. During pregnancy, a child may be exposed to certain factors (e.g., pollutants, toxins, vaccines as well as nutritional factors including, a lack of essential minerals such as calcium, zinc, iodine and potassium (London, 2000) that could increase the risk of developing ASD. Scientists have focused attention to increased incidence of autoimmune disorders, impaired immune systems, immunoglobulin deficiencies and increased auto antibodies to brain antigens, as well as, nonspecific IgG antibodies against brain tissues (Chez *et al*., 2004). Zimmerman *et al*. (2006) believed that maternal antibodies that recognize fetal brain antigens might therefore alter brain development normally. Currently, research shows that the number of antibody-producing B cells was increased by 20% in the autism (Amaral *et al*., 2008). Beside, the immune system appeared to be deregulated which provided some support for the report that more time should be spent and more attention should be given to immune factors as contributors to autism. Martin *et al*. (2008) reported reactivity of maternal serum against both human and monkey fetal brain proteins. They went further in their studies and reported that the neuropathology of ASD is still in the infancy and yet, cerebellar irregularity is one of the most consistent findings in the brains of these subjects. In recent years, considerable epidemiological studies and public attention were given to the theory that the measles-mumps-rubella vaccine potentially contributed to the development of autism due to the mercury-containing thimerosal used as a preservative (Madson *et al*., 2002).

An important hypothesize reported that autism may be caused by mitochondrial dysfunction (Chauhan and Chauhan, 2006; Rossignol and Bradstreet, 2008) and individuals with autism have overall lowered cellular energetic and scarce reserve mitochondrial energy capacity that could lead to cognitive impairment, language deficits and abnormal energy metabolism. Damaged mitochondria not only produce more oxidants but also vulnerable to oxidative stress and it was suggested that there is a disturbance of energy metabolism in the brain of autistic patients (Chauhan and Chauhan, 2006). They also added that oxidative stress is associated with premature aging of cells and can lead to tissue inflammation, damaged cell membranes, autoimmunity and cell death. Diagnosis of autism is difficult because of the variability with which the disorders discernible themselves.

Since, Red Blood Cells (RBCs) are the essential elements in providing the body cells (including Purkinje cells) with oxygen, through which energy is provided to the cells and due to the fact that White Blood Cells (WBCs) are the responsible elements for the production of antibodies, in the present study an attention given to assess the diagnostic features of autistic children and their mothers' peripheral blood. Structure of leukocytes specifically may shed light on the role of immune factors as contributors to autism.

MATERIALS AND METHODS

Participant: Fifteen autistic children, including 9 males ranging from 7 to 19 years old and 6 females ranging from 5-12 years old examined. The children were labeled as having autism according to the American Psychiatric Association Criteria (APA, 1996) (Table 1). Mothers of autistic candidates were investigated.

Procedures follow for blood preparations: Blood samples were obtained from the fingers of the above mentioned participants. Samples obtained were divided into two parts for both light and ultrastructure studies. To prepare a blood smear for light microscopic examination Gurr (1953) method was used. A drop of blood was spread thinly on a glass slide dried, fixed with methyl alcohol

Table 1: According to the APA (1996), a person could be labeled as having autism, if he or she meets the following criteria

A total of six (or more) items from (A), (B) and (C) with at least two from (A) and one each from (B) and (C)
A: Qualitative impairment in social inter action, as manifested by at least two of the following:
• Marked impairment in the use of multiple non-verbal behaviors such as eye-to-eye gaze, facial expression, body postures and gestures to regulate social interaction
• Failure to develop peer relatiouships appropriate to developmental level
• A lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing bringing, or pointing out objects of interest)
• Lack of social or emotional reciprocity
B: Qualitative impairment in communication as manifested by at least one of the following:
• Delay in or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
• In individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
• Stereotyped and repetitive use of language or idiosyncratic language
• Lack of varied, spontaneous make-believe play or social imitative play appropriate to development level
C: Restricted repetitive and stereotyped patterns of behaviour, interest and activities, as manifested by at least one of the following:
• Encompassing preoccupation with one or more stereotyped and restricted patterus of interest that is abnormal either in inteusity or focus
• Apparently inflexible adherence to specific, nonfunctional routines or rituals
• Stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)
• Persistent preoccupation with parts of objects
Delays or abnormal functioning in at least one of the following areas, with ouset prior to age 3 years
• Social interaction,
• Language as used in social communication, or
• Symbolic or imaginative play
The disturbance is not better accounted for by Rett's disorder or childhood disintegrative disorder

and stained with "Leishman's stain" to reveal various blood cell types. For electron microscopy, 5 mL heparinized blood samples from autistic children and their mothers were collected and centrifuged for 20 min at 2500 rpm. A thin white buffy coat was formed between erythrocyte below and plasma above. To avoid disturbance of the Buffy coat, very gently, 2 cc. buffered $_4F_1G$ (4% formalin and 1% glutaraldehyde) was added, using a pipette in a sloping position to allow the fixative to run down the side of the tube. The tube was then covered and allowed to stand for 18 h at 4°C. 0.1 M phosphate buffer (pH 7.4) was added into the test tube containing the precipitate free cells, the tube was shaken again and the mixture was rinsed to remove the fixative sticking to the free cells. After that, centrifugation was performed and the supernatant liquid was discarded. An L-shaped needle was inserted down the side of the tube to a position several millimeters below the puffy coat plug. It was then moved around the wall of the tube to loosen the plug from the tube and from the red cells below. The plug was then pulled out of the tube and placed in buffer solution. A thin l mm slice was cut post-fixed in 1% OsO_4, then trimmed into smaller pieces and processed as ordinary tissue. The buffy coat was dehydrated in a series of increasing concentrations of acetone (10 min each). Dehydrated specimens were embedded in Epon-Araldite resin mixture. Ultrathin sections (50 nm thick) were double stained with uranyl acetate for ½ h. and lead citrate for 20-30 min and were examined with a Joel 100 CX electron microscope (Reynolds, 1963).

RESULTS

Light micrographs of peripheral blood of autistic children (Fig. 1a-f) showed numerous morphological changes in the structure and size of blood cells. Concerning erythrocytes it was noticed that as in normal preparations RBCs (6 μm diameter) were very numerous and lack nuclei. The first imposing pathological change however, was the presence of echinocytes (identified by 5-10 spicules/ erythrocyte, scattered randomly over the surface (Fig. 1a-d). Worthy to mention, that these cells were almost absent in the normal individuals. Also, numerous morphological altered RBCs including; anisocytosis (with abnormal size variation) (Fig. 1b), poikilocytosis (with abnormal shape variation) (Fig. 1b, c), a special poikilocytotic erythrocytes (keratocytes or horn cells appeared as half-moon or spindle shaped cell) (Fig. 1c, d), dacrocytes (Fig. 1a, e and f) (with a profound increase in the number of teardrop shaped erythrocytes), schistocytes (fragmented RBCs with a variety of shapes and size (Fig. 1c and d), elliptocytes (characterized by an elliptical shape), Pita bread cells (Fig. 1e) characterized by their elongated folded shape), Bite Cells (RBCs appear as if bitten a piece off) (Fig. 1f) in addition to some erythrocytes bounded together in "Rouleaux" shape, resembling coins stacked to each other Profound examination of blood preparations obtained from autistic children showed that ultrastructurally the erythrocytes reveal abnormal morphological variation, similar to that mentioned before. In addition, our preparations showed that although there were normal appearing leukocytes, in the blood of autistic children several altered leukocytes including granulocytes and agranulocytes were observed. The morphological alterations included the size, shape and position of the nuclei. Worth mentioning that alterations among leukocytes were not easily detected using light microscope. Light preparations of the blood of autistic children showed only, some leukocytes own irregular hyperchromatic nuclei while others appear clefted or vacuolated or invaginated. In addition, centralization of the monocyte (Fig. 1e, f) nuclei vacuolization of leukocyte cytoplasm was observed.

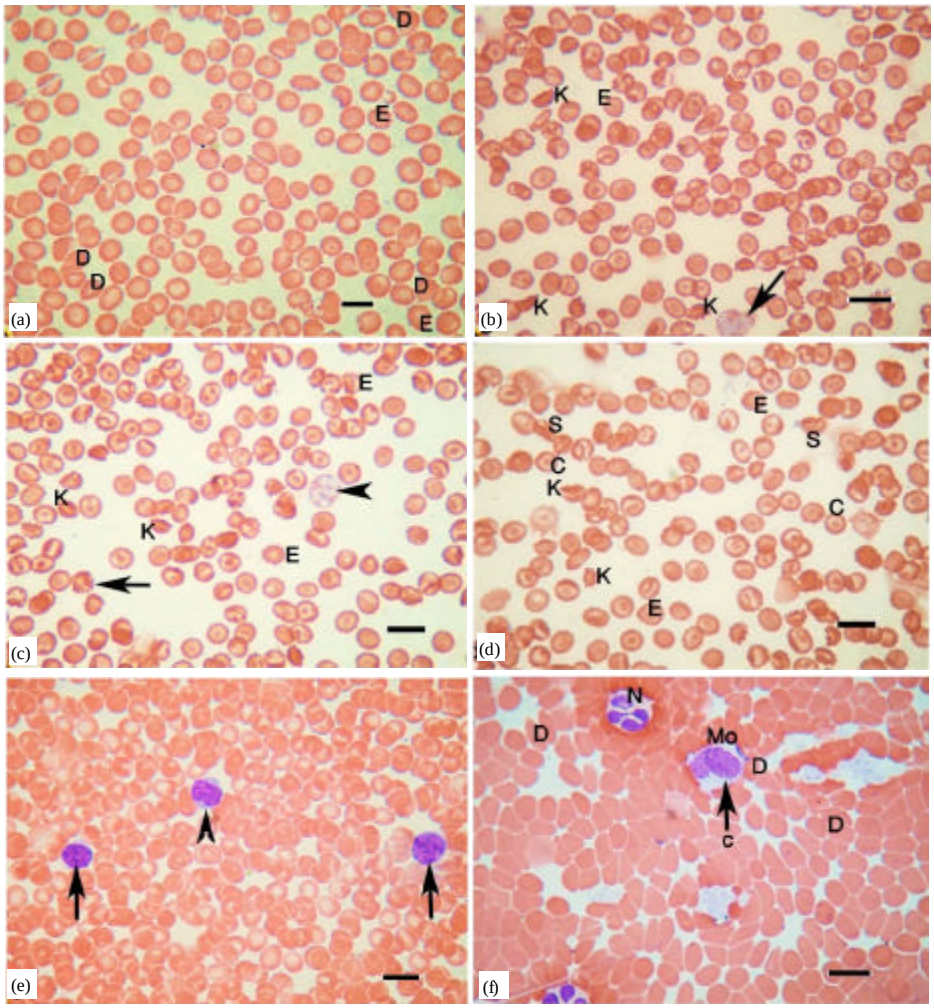

Fig. 1(a-f): Light micrographs of peripheral blood smears of autistic children, (a) Dacrocytes (D), echinocytes (E) with 5-10 spicules, (b) Anisocytosis with different sizes, poikilocytosis with irregular morphology, echinocytes (E), half-moon or keratocyte (K), granulocyte with irregular cell boundary (arrow) and enlarged multilobes, (c) Poikilocytosis, echinocytes (E), schistocytes, fragments (arrow) and half-moon or spindle shaped keratocyte (K), leukocytes with polymorphic nucleus (arrowhead), (d) Echinocytes (E), the half-moon keratocytes (K), schistocytes, fragments (S) and codocytes or Bull's eye (C), (e) Dacrocytes or teardrop shaped, echinocytes, granulocyte with normal morphology (bold arrow) and (f) Monocyte (Mo) with clefted nucleus (C), neutrophil with a multilobed nucleus (N), dacrocytes (D), hypertrophied macrocytes RBCs, Methanol fixed-Leishman's stained, Scale bars: 10 μm

Peripheral blood cells of mothers of autistic children (Fig. 2a-f), showed analogous alterations to that observed in preparations of their children. Irregular shaped RBCs (poikilocytosis) (Fig. 2c and f), RBCs bounded together in " Rouleaux shape" (Fig. 2a, b and g), echinocytes

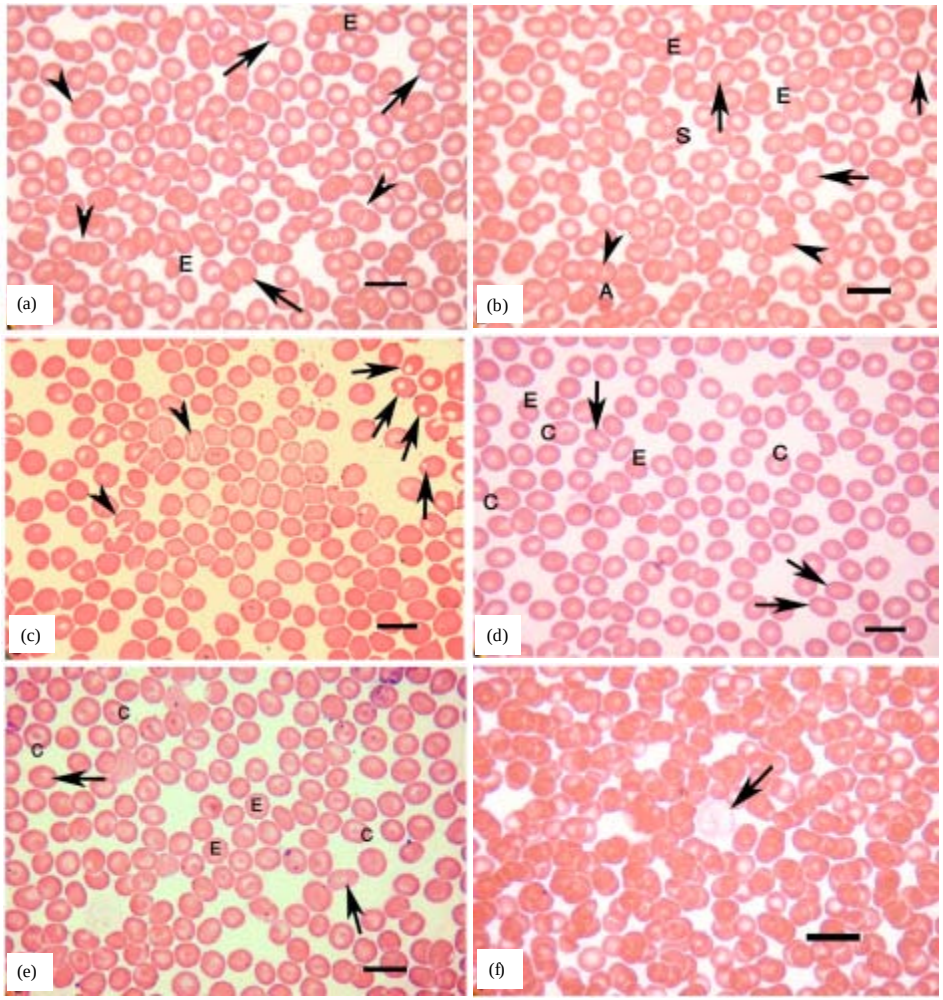

Fig. 2(a-f): Light micrographs of peripheral blood smears of mothers of autistic child, (a) RBCs with different morphology, echinocytes with spicules (E), macrocytes (arrows) and rouleaux RBCs (arrowheads), (b) RBCs with different morphology, speculated echinocytes (E), macrocytes (arrows), rouleaux (arrowheads), elongated shape stomatocyte (S) with unevenly distributed hemoglobin and acanthocyte (A) with thorn shaped membrane protrusions, (c) RBCs with different morphology, macrocytes, poikilocytosis (arrowheads) and some stomatocytes with uneven distributed hemoglobin (arrows), (d) RBCs in different orientations, elliptocytes (arrows), echinocytes (E) and codocytes or bull's eye (C), (e) Echinocytes (E), bull's eye codocytes (C) and elliptocytes (arrows) and (f) Poikilocytosis, rouleaux, monocyte with central nucleus (arrow), Methanol fixed-Leishman's stained, Scale bars: 10 μm

(Fig. 2, a, b, d, e) and thorns (acanthocytes) (Fig. 2b), teardrop shaped RBCs (dacrocytes). Some erythrocytes showed other abnormalities including macrocytes (Fig. 2a-c) stomatocytes (Fig. 2b and c) with unevenly distributed hemoglobin, codocytes (Fig. 2d and e) which indicate RBCs resembles a bull's eye as well as, elliptocytes (Fig. 2d) were also noticed. On the other hand,

leukocytes morphological features were not clearly detected, since the magnification of the light microscope was not sufficient to resolve the details. Consequently, lymphocytes, with euchromatic nuclei, others appeared hyperchromatic, or with cleaved nuclei or with abnormally distributed chromatin were observed. Monocytes (Fig. 2f) with displaced central nuclei were also observed in these preparations. In general, the results of the electron micrographs of peripheral blood cells confirmed the alterations observed in the light microscope in the autistic candidates concerning the erythrocytes as well as the leucocytes.

Electron micrographs of the leukocytes of autistic candidates (Fig. 3 and 4a, c) reveal interesting changes. Monocytes with kidney-shaped decreased size nuclei and marginated heterochromatin were noticed (Fig. 3a). One of the most important feature which was observed in cytoplasm of such cells, was the absence of the mitochondria and even those which had this organelle, the mitochondria were vacuolated with indistinct membrane and abnormal distribution. Also, numerous primary and secondary lysosomes, multivesicular bodies, large size vacuoles and myelin figure noticed in the cytoplasm. Moreover, it was observed that the lymphocytes (Fig. 3c) seemed to have several anomalies, their nuclei decrease in size in most of them, thus, the karyoplasmic ratio was altered. Moreover, their nuclei were eccentric marginated heterochromatin. It is impressive to mention the presence of nuclear pocket type I (Fig. 3c) in some of them. The inner nuclear membrane appeared disrupted in some lymphocyte. Some of the lymphocytes showed evagination of the perinuclear space toward the inside of nucleus (Fig. 3c); this may be the first step in the formation of nuclear pocket structure; while in others the outer nuclear membrane is evaginated to the outside. The cytoplasm of most lymphocytes lack mitochondria (Fig. 4a), others that contain mitochondria they have it vacuolated (Fig. 3a). In some cases, myelin figure is observed in the cytoplasm and while in others appeared devoid of organelles. In addition, cleaved furrow (Fig. 4a) was observed in the cytoplasm of some cells. Moreover, the cell boundary was irregular in some of the lymphocytes. On the other hand, the electron micrographs of the present study showed cells resembling to great extent to the plasma cells. A major abnormality was the inversed distribution of the chromatin material in the nucleus (Fig. 5a, b). Also, remarkable was the central round nucleus and the complete absence of rER. Mitochondria were mostly disrupted. The hypertrophied Golgi complex was detected. The cytoplasmic organelles were abnormally distributed and dilation of the perivisceral nuclear space was observed and the plasma membrane was ruptured in others.

Autistic candidates blood reveals, basophiles with normal morphology; with bilobed nucleus, obvious nucleolus in each lobe; and normal morphological appearing granules that were normally distributed. They possess small and large size granules with dense core and few mitochondria with dense matrix and indistinct membranes. The cytoplasm contains numerous ribosomes and scarce ER. In addition, basophiles with altered structure also observed. Their nuclei appeared hyperchromatic and marginated. The cytoplasm also, contains small size vacuolated mitochondria as well as basophilic granules of different sizes where some of them seem to be released out of the cytoplasmic membrane. Ultrastructural studies also revealed granulocyte but their type could not clearly identify due to the intense alteration in their morphology. In some cases the nucleus was highly euchromatic and the perivisceral nuclear space was dilated. The plasma membrane was ruptured and their cytoplasm contains autophagic vacuole with typical double membrane, as well as numerous different sized granules. These possess different electron densities and vacuolated mitochondria.

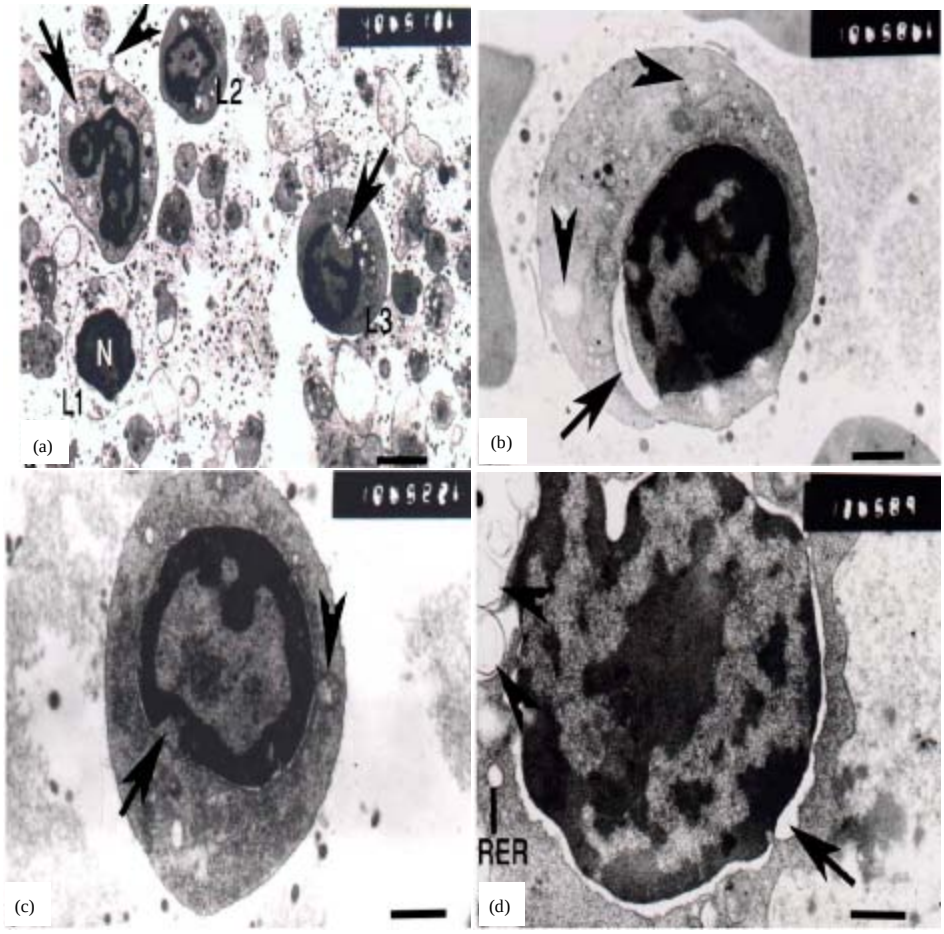

Fig. 3(a-d): Electron micrographs of (a, c) Leukocytes of autistic children, (b, d) Leukocytes of mothers of autistic children, (a) Monocyte with kidney-shaped hyperchromatic nucleus, vacuolated mitochondria, vacuoles containing myelin (line arrow), Arrowhead: Short pseudopodia, lymphocyte (L1) with a small size nucleus (N), marginated heterochromatin, vacuolated mitochondria, lymphocyte (L2) possesses a small size hyperchromatic nucleus, and the cytoplasm possesses vesicles with dense core granules, Lymphocyte (L3) with eccentric nucleus containing nuclear pocket (bold arrow), vacuolated mitochondria. A large number of small platelets containing small granules in their core, (b) Altered lymphocyte, eccentric nucleus hyperchromatic, membrane blebbing (bold arrow). Arrowhead: Vacuolated mitochondria, the cytoplasm containing ribosome, RER small transverse vesicle, black arrow: Vacuolated cytoplasm, (c) Lymphocyte with central nucleus, with marginated chromatin, perinuclear space evaginated toward the inside of nucleus; nuclear pocket (line arrow), small and large mitochondria adhering nuclear envelope (arrowhead) and (d) Altered lymphocytes with compact nucleus, marginated heterochromatin, perinuclear space dilated at certain sites forming blebs (arrow). The cytoplasm house scanty free ribosomes, RER vesicles and myeloid bodies (arrowheads), $_4F_1G$ fixed and uranyl acetate and Lead citrate stained, Scale bars: (a) 2.5 , (b, c) 1 and (d) 0.72 μm

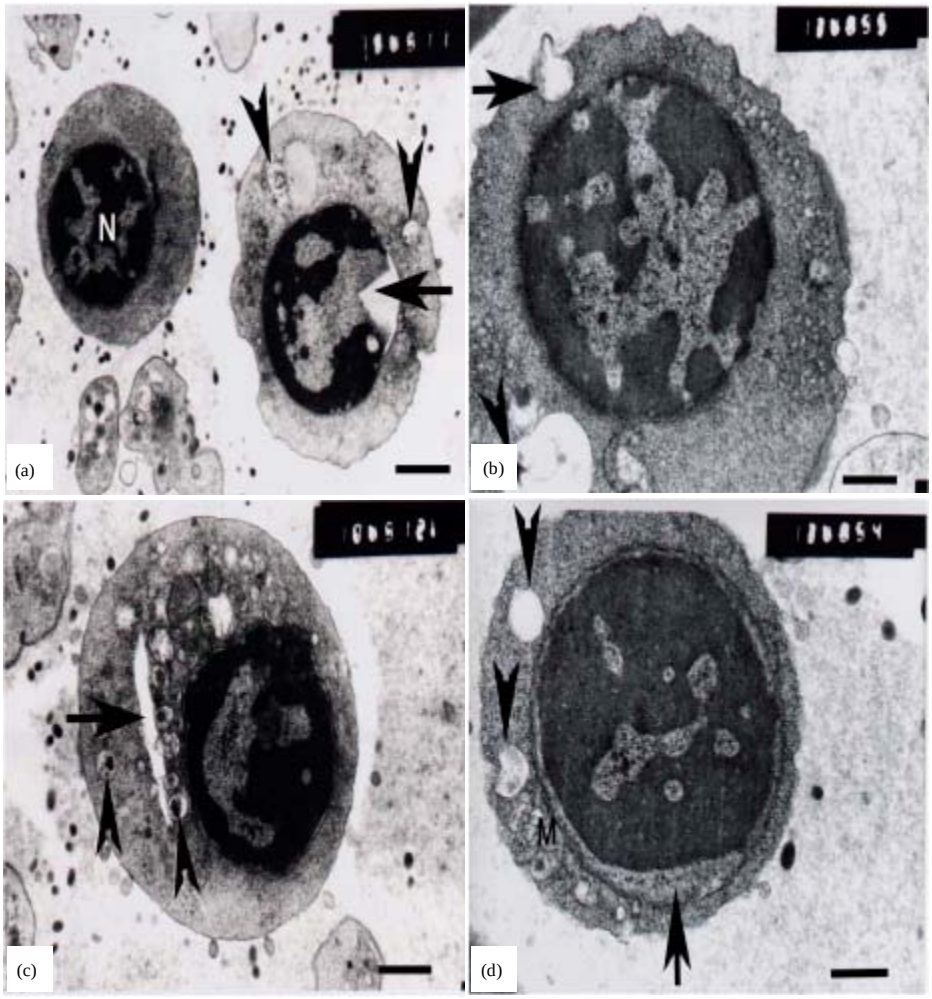

Fig. 4(a-d): Electron micrographs of (a, c) Leukocytes of autistic children and (b, d) Leukocytes of their mothers, (a) Lymphocyte with somehow normal morphology, hyperchromatic nucleus (N), huge amount of ribosome, complete absence of mitochondria, Lymphocyte at left side with large size, marginated chromatin; the inner nuclear membrane disrupted (arrow), few vacuolated mitochondria (arrowhead), (b) Altered lymphocytes, hyperchromatic nucleus, heterochromatin arranged directly underneath the nuclear membrane, housed ribosomes, RER vesicles, ruptured vacuole (arrow) and secondary lysosomes (arrowhead), (c) Spherical lymphocyte, with small size hyperchromatic eccentric nucleus with slightly irregular nuclear envelope, with distinct perinuclear space, the cytoplasm possesses light vesicle with a dense core (arrowheads), cleaved cytoplasmic furrow (bold arrow) and (d) Altered lymphocyte with compact heterochromatin, displacement of heterochromatin to the interior leaving an area around it possessing euchromatin only (arrow), the cytoplasm scanty composed largely of free ribosome, RER vesicles (arrowheads) as well as vacuolated mitochondria (M), $_4F_1G$ fixed and uranyl acetate and lead citrate stained, Scale bars: (a) 1.34, (b, c) 2 and (d) 0.72 μm

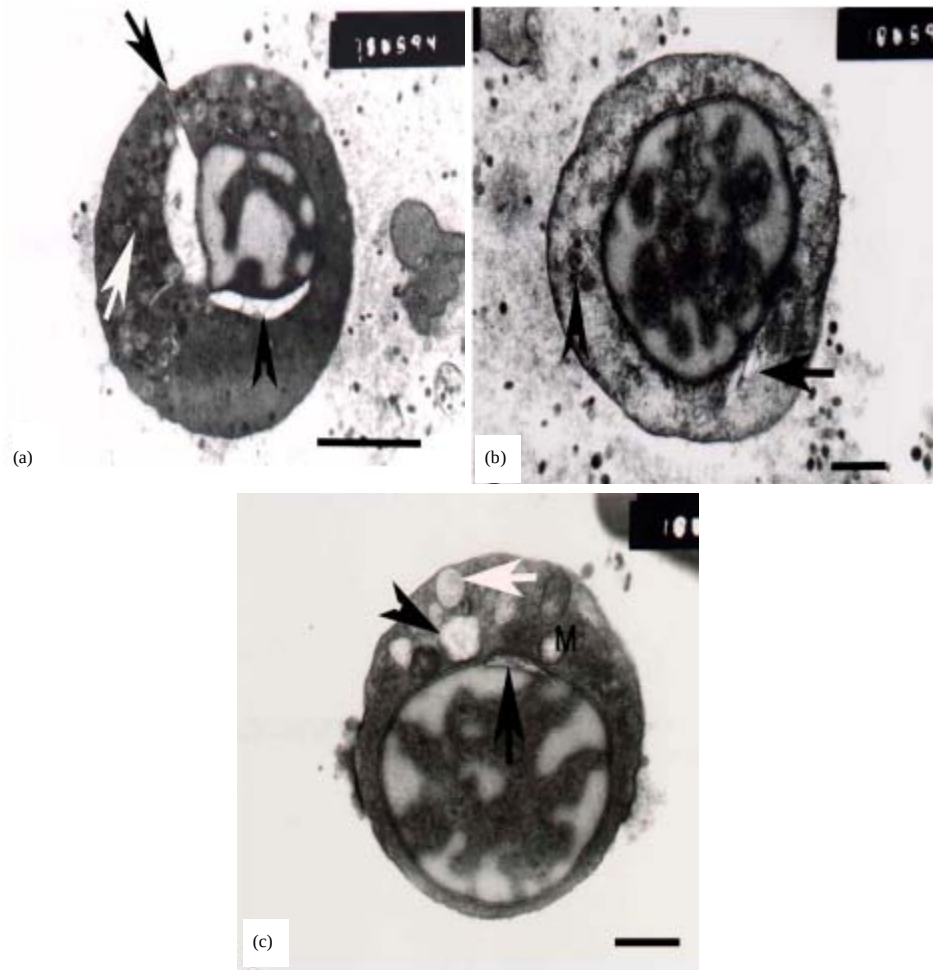

Fig. 5(a-c): Electron micrographs of (a, b) Leukocytes of autistic children and (c) Their mothers, (a) Plasma cell with a ruptured plasma membrane (arrow), with altered size and shape, central nucleus, highly dilated perinuclear space (arrowhead), numerous mitochondria, small dense granules (white arrows), (b) Plasma cell with a central large size nucleus, with a decreased chromatin distribution that appear altered; dilated Golgi cisternae (arrow), a large number of small sized vesicles; arrowheads pointing at large vesicle containing granules and (c) Highly altered plasma cell, the nucleus showed altered chromatin distribution, the heterochromatin shifted to inside instead of being arranged directly underneath the nuclear membrane. Note also, nuclear pocket type I (arrow), the cytoplasm scanty composed largely of ribosome, loss of mitochondrial ultrastructure (M), multivesicular body (arrowhead) and autophagosome (white arrow), $_4F_1G$ fixed and uranyl acetate and lead citrate stained, Scale bars: (a) 1.34 and (b, c) 2 μm

Worthy to mention that platelets also showed morphological alterations that were not identified with a hight microscope. Their plasma membrane was ruptured, their matrix was cleaved and their granules seem to be released out. Their plasma contains central microfilaments and vacuolated mitochondria (Fig. 6a, b).

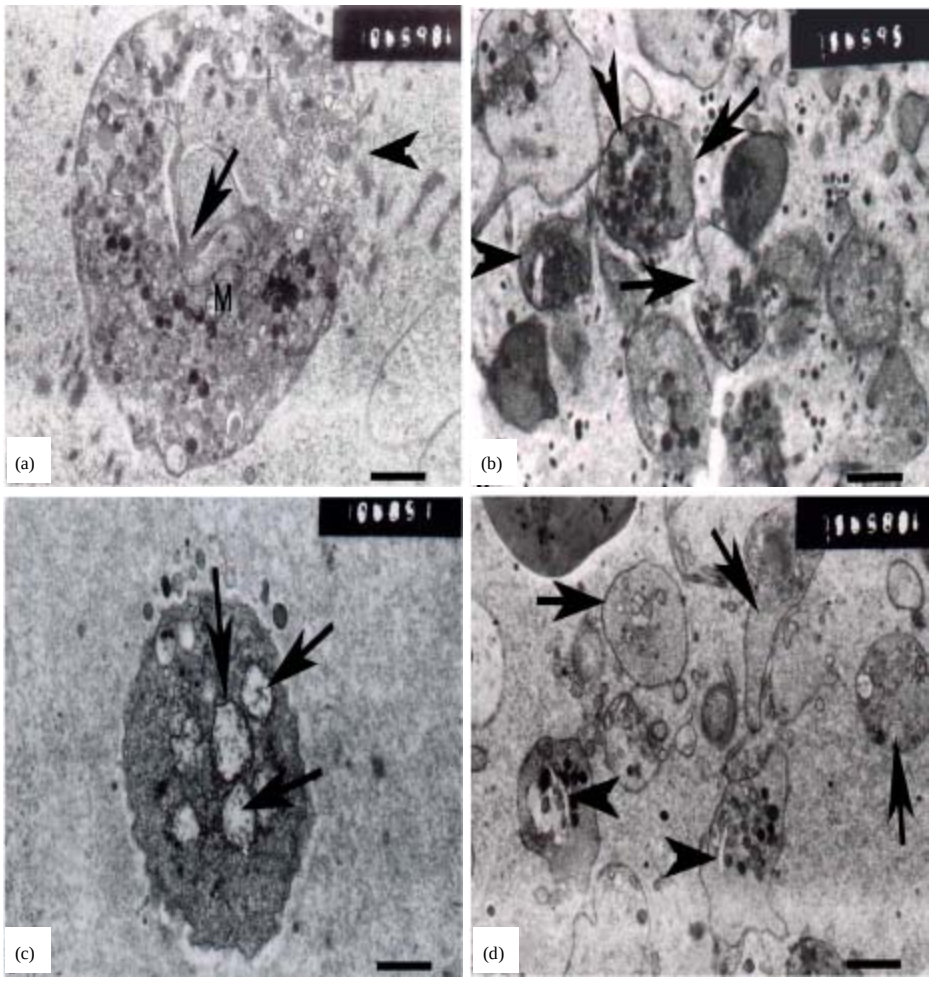

Fig. 6 (a-d): Electron micrographs of (a, b) Altered platelets of autistic children and (c, d) Their mothers, (a) Platelets with ruptured plasma membrane (arrowhead), granules of different densities in the matrix, dense bodies, Golgi cisternae (arrow), vacuolated mitochondria (M), (b) Platelets of different morphology with ruptured plasma membrane (arrows), marginal bundle of microtubules are absent, a decrease in the amount of granules in some and their release into the matrix, while others possess numerous small size granules (arrowheads), (c) Platelet with dense matrix, irregular plasma membrane, some of the granules released out, Vacuolated mitochondria (arrows), light granules in the center of the platelet and (d) Different shapes and size of altered platelets, Marginal bundle of tubules in all are absent, The matrix is scant and there is a reduced number of granules while others lack granules (arrows), Cleaved furrow in the matrix in some is present (arrowhead), $_4F_1G$ fixed and uranyl acetate and lead citrate stained, Scale bars: (a, c) 2 and (b, d) 1.34 μm

As noticed in light micrographs, electron micrographs of the mothers of autistic children (Fig. 3 and 4b and d) revealed comparable alterations. The most important observation in almost

all lymphocytes was the decrease in the size of their nuclei. Thus, the karyoplasmic ratio was altered in the lymphocytes. In addition, the nuclei were eccentric in position with marginated heterochromatin. Beside, reduced size and eccentric nucleus some lymphocyte possess heterochromatin that was shifted to inside instead of being marginated. Nuclear pocket type I and membrane blebbing was observed among lymphocytes of these preparations. On the other hand, it was found that displacement of heterochromatin to the interior leaving an area around it possessing euchromatin only observed in some lymphocyte; while in another, the nucleus is compact and the perivisceral space appears dilated at certain sites forming blebbing (Fig. 3b, d). The cytoplasm of most cells appears scant, composed largely of monosomal ribosome and rough endoplasmic reticulum. The mitochondria were absent in many cells and scarce in others. In these cells that contain mitochondria they have it vacuolated. Also, the cytoplasm of some of the lymphocytes showed numerous light vesicles with dense core granules. The electron micrographs of the mothers also showed alterations in the granulocytes morphology which involved both the nucleus and the cytoplasmic organelles. Ultrastructurally, the granulocytes were altered to a great extent; so that it was practically impossible to identify their exact type. In all granulocytes, rupturing of the plasma membrane was noticed. In addition, the cytoplasm contained light and dark granules but lacked mitochondria. In some cells that possessed mitochondria, they were vacuolated. Granulocytes revealed also that the chromatin material condensed into a u-shape eccentric form. In some other granulocyte, the nucleus was hyperchromatic and the chromatin material was marginated. There is formation of nuclear pocket type I (Fig. 5c) showing myelin filaments. Granulocytes, with ruptured nuclear membrane and highly euchromatic nucleus also observed. A basophile with small size nucleus and compact heterochromatin arranged directly underneath the nuclear membrane also observed among these preparations.

It was found also that blood of the mothers of autistic children; with alterations comparable to that observed in the platelets of the peripheral blood of the autistic children (Fig. 6c, d). Their plasma membranes were ruptured, their matrix was cleaved, their mitochondria were vacuolated and the granules were released out. Some platelets contained numerous myeloid bodies.

DISCUSSION

Factually, blood serves as the most convenient indicator of the general condition of the animal body. Subsequently, hematological studies are promising tools for investigating physiological changes caused by different factors (McCarthy *et al.*, 1971). Up to the present, however, information about the diagnostic features of blood of autistic children is not available yet. Thus, the following diagnostic features described for the first time in the peripheral blood of the autistic children.

During the course of this investigation, significant morphological changes were observed in the cellular elements of the peripheral blood of the autistic children. Data of the present study showed that microscopic findings correlated well with ultrastructure observations concerning the blood of autistic children and their mothers, where both revealed echinocytic erythrocytes (with equally-spaced projections on the membrane). Beutler *et al.* (1995) described similar alterations in case of Uremia, pyruvate kinase deficiency and in case of liver disease. Our preparations showed also, RBCs with anisocytosis and poikilocytosis. Similar morphological observation was reported previously by Thompson (1983) who reported that in anemia, although there is usually a little variation in the size and shape of the erythrocytes, there is sometimes quite marked anisocytosis and poikilocytosis with a moderate degree of macrocytosis. Lynch (1990) related echinocytic

erythrocytes in pyruvate kinase deficiency form, to decreased ATP generation resulting in loss of water and potassium from RBC. We are in agreement with Lynch (1990) since the presence of altered mitochondria in different types of WBCs indicated decreases in ATP.

Moreover, one of the most striking abnormalities observed in our preparations include RBCs stacked over each other (Rouleaux). This is in agreement with the previous study reported early by Lessin *et al.* (1976) who related Rouleaux feature to the high serum protein concentrations. Moreover, teardrop-shaped erythrocytes (dacrocytes) were observed in both light and electron micrographs. The results of the present study are compatible with the morphological alterations reported previously in patients with myelofibrosis and thalassemia (Bessis, 1972). Keratocyte, characterized by a half-moon or spindle- shaped is another morphological alteration observed. Similar alterations were reported by Beutler *et al.* (1995) in patients with disseminated intravascular coagulation or a vascular prosthesis. The preparations also, revealed ellipsoid erythrocytes with a central area of pallor and hemoglobin at both ends of the cell referred to as elliptocytes. Schwartz and Stansbury (1954) associated the infiltrative disorders of the bone marrow to the formation of elliptocytes. Lee *et al.* (1993) believed that a large number of elliptocytes usually indicates hereditary elliptocytosis while moderate numbers of elliptocytes are seen in thalassemia and myelofibrosis and lesser numbers in iron deficiency and hypersplenic states.

In addition to the aforementioned morphological alterations, erythrocytes with increased surface area to volume ratio appearing as target cells with bull's eye (codocytes) were observed. These results are in accordance with alterations reported in patients with liver disease, iron deficiency, post-splenectomy, decreased lecithin cholesterol acyl-transferase activity (Bessis, 1977). Bessis *et al.* (1983) added that lesser numbers of target cells are found in sickle cell anemia, iron deficiency and lead intoxication. During the course of the present study, it was clearly noticed that comparable morphological alterations to that found in autistic children were observed in the light micrographs of the peripheral blood smears of their mothers.

Examination of the peripheral blood smear with light microscope is an inexpensive and rapid diagnostic tool to examine morphological alterations in the blood cells. However, it does not provide reliable access to information about a variety and detailed information of hematologic disorders. In this investigation, light microscope provided us with a general and a rough view of the alterations that have occurred at the level of the leukocytes and did not provide us with detailed alterations at the level of organelles within the cells. So, it was of great interest to use an electron microscope to examine the peripheral blood of autistic children and their mothers to identify and verify the morphological alterations which were roughly observed with the light microscope. However, due to the resolving power of the electron microscope, our preparations clearly demonstrated more details concerning leukocytes.

One of the most important and striking alterations among all leukocytes types is the vocalization of the mitochondria. Ghadially (1985) described similar alterations in leucocytes of monocytic leukemic patients. It is well known that mitochondrial functional or structural abnormality is one of the most sensitive indicators of cell injury (Roodyn and Wilkie, 1968). Zafar *et al.* (1982) found that structural damage to the mitochondria in mammals was specifically attributed to inhibition of mitochondrial protein synthesis. Moreover, Lombard (1998) postulated that autism is greatly related to mitochondrial dysfunction. He also, added that areas of increased metabolic activity, specifically in the developing brain, are likely more susceptible to mitochondrial dysfunction. Lombard (1998) confirmed his hypothesis by neuro-imaging procedures including positron emission tomography scanning and nuclear magnetic resonance spectroscopy. He related

the mitochondrial dysfunction in autism to lactic acidosis. He also stated that the precise biochemical abnormality has not yet been identified. Similarly, Chauhan and Chauhan (2006) suggested that abnormal energy metabolism in autism is due to mitochondrial dysfunction where, they added, damaged mitochondria produce more oxidants and are vulnerable to oxidative stress. In addition, Weissman *et al.* (2008) reported that the most common ultrastructural abnormalities in autism spectrum disorder were abnormal mitochondrial morphology and increased number of mitochondria. They also added that defective mitochondrial oxidative phosphorylation is an additional pathogenetic basis for a subset of individuals with autism (Weissman *et al.*, 2008).

It is worth to mention that Heggtveit (1969) related alterations of the mitochondria to hypoxia as well as to ischaemia which lead to the degeneration of the cell. On the other hand, Kroemer *et al.* (1998) related dysfunction or damaged mitochondria to apoptosis. They reported that the consequences of mitochondrial dysfunction (collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione and release of soluble intermembrane proteins) entails a bioenergetics catastrophe culminating in the disruption of plasma membrane integrity (necrosis) and/or the activation of specific apoptogenic proteases (caspases) by mitochondrial proteins that leak into the cytosol (cytochrome C, apoptosis-inducing factor) with secondary endonuclease activation (apoptosis). Kroemer *et al.* (1998) added that, the relative rate of these two processes (bioenergetics catastrophe versus protease and endonuclease activation) determines whether a cell will undergo primary necrosis or apoptosis. Recently, studies have pointed to a subset of autism associated with the biochemical endophenotype of mitochondrial energy deficiency, identified as a subtle impairment in fat and carbohydrate oxidation (Gargus and Imtiaz, 2004). In addition to mitochondrial abnormalities, electron micrographs of the peripheral blood of the autistic children revealed irregularly shaped monocytes with irregular, hyperchromatic nucleus and with pleomorphic granules in the cytoplasm. Dickerson (2000) observed hyperchromatic monocytes in mammalian monocytic leukemia. Also, Ghadially (1985) stated that lymphocytes were found with irregular hyperchromatic nuclei and plasma membranes with numerous filopodia in monocytic leukemic patients.

Observations of the electron micrographs of the monocytes of the autistic children revealed also the presence of myelin figures in the cytoplasm. Hansson *et al.* (1997) reported similar findings in monocytes in cases treated with tricyclic antidepressant drugs used for the treatment of manic-depressive disorders.

Morphological alterations observed in the peripheral blood cells of the autistic children also include lymphocytes with diminished size nucleus and abundant marginated heterochromatin. Walther *et al.* (2009) reported the presence of hyperchromatic lymphocytes in a study of 6 cases of chronic lymphocytic leukemia that incidentally involved 6 exceptional specimens for biopsy-proven carcinoma.

Examination of electron micrographs of the lymphocytes of the peripheral blood of the autistic children, revealed evagination of the nuclear membrane and the presence of nuclear pockets in fewer numbers of cells. It is known that nuclear pockets are produced by folds or ruffles arising from the surface of the nucleus which entraps cytoplasmic material (in case of type I) or nuclear material (in case of type II). Similar observations were mentioned by Conforti *et al.* (1978) in human lymphocytes treated with antimitotic substance which is known to produce nuclear changes *in vivo* and *in vitro*. Similarly, the ultrastructural investigation performed by Stekhoven and

Holland (1986) revealed that the nuclei of the lymphoid cell population of bone marrow and blood of children with acute lymphoblastic leukemia regularly showed the presence of two types of nuclear pockets and nuclear clefts.

Further observation of the peripheral lymphocytes of the autistic children in our study revealed cleavage of the cytoplasm. Nano *et al.* (1996) reported that high-dose of interleukin-2 plays a crucial role in the morphological features of the lymphocytes causing the nucleus to be asymmetrically located at the periphery of the cell and characteristic cleavage of the cytoplasm with the formation of many blebs. Other alteration observed in the lymphocytes of the autistic children was the reduced karyoplasmic ratio where the size of the nucleus was highly reduced. Hovsepian and Frenster (2003) reported similar observations in lymphocytes which were cultured for 72 h in Phytohemagglutinin free medium. They also added that the nucleus of these lymphocytes were in heterochromatic state. In general, the electron micrographs revealed nuclei of reduced size and with highly endowed heterochromatic content, with dilated perinuclear space and formation of large blebs, as well as nuclear pockets. All the previously mentioned features are known as signs of apoptosis, as reported by Ghadially (1985).

An interesting observation of the peripheral blood of the autistic children was the presence of the plasma cells which are not usually present in the peripheral blood of normal cases. These cells also showed alterations including dilated perivisceral nuclear space and showed that the chromatin material in the nucleus had an inverted distribution (i.e., the cartwheel heterochromatin appearance is lost). It is well known that normal plasma cells are large size lymphocytes with a considerable karyoplasmic ratio and a characteristic nuclear appearance (Ghadially, 1985). These cells identified with their basophilic cytoplasm and house eccentric nucleus with characteristic distributed heterochromatin in the form of cartwheel arrangement. The surrounding cytoplasm possesses a light area which upon examination showed a well developed Golgi complex and abundant Rough Endoplasmic Reticulum (RER).

Considering the granulocytes in our electron micrographs of the peripheral blood of the autistic children, they were highly altered that it was practically impossible to identify their type. Some of the granulocytes showed hyposegmented mononucleus. The cells showed characteristic apoptotic features in both the nucleus and the cytoplasm. The nucleus showed compact and segregated chromatin sharply delineated toward the periphery of the nucleus. Marked condensation of the cytoplasm with distorted organelles and mild convolution of the cellular outlines were noted. Similar alterations were described by Shetty *et al.* (2001) and referred to pseudo Pelger-Huet disorder. Moreover, granulocytes of bilobed non-segmented hyperchromatic nucleus were observed. Cunningham *et al.* (2009) described similar alterations in neutrophils which had intensely stained and prominent nuclei found in the peripheral blood smear of patients affected by Pelger-Huet anomaly. They also observed bilobed hyperchromatic neutrophils in patients affected by myelodysplastic disorder.

Morphological alteration observed in the light micrographs of peripheral blood of autistic children also revealed alterations in the platelets, included ruptured plasma membrane, granules of different densities, vacuolated mitochondria and displaced or lack of microtubules. Alterations at the level of the platelets in autistic candidates was mentioned by Anderson *et al.* (1990) and related to hyperserotonemia in autism. Although they did not specify the detailed alteration in the platelets, they suggested that the planned and ongoing studies of platelet function and composition would allow to better define the alteration which they presume to be present in the platelets of autistic subjects. Similarly, Janusonis (2005) related alterations of the platelets in autistic subjects

with elevated levels of 5-hydroxytryptamine. He added that the early developmental alteration of the autistic brain and the autistic platelet hyperserotonemia may be caused by the same biological factor expressed in the brain and outside the brain.

Interestingly, the morphological alterations observed ultrastructurally in the peripheral blood of the autistic children were also observed in the blood of their mothers. But in addition to the mentioned alterations, peripheral blood of the mothers showed the presence of blebs in the nucleus. Ahearn *et al.* (1974) reported that DNA-inhibiting chemotherapeutic agents can induce the formation of nuclear blebs. They added that during the active phase of the acute leukemia, there is a definite correlation between aneuploidy and a high frequency of nuclear blebs in the peripheral blood cells due to the lack of Vitamin B12 in autistic candidates necessary for the synthesis of thymine and subsequently, DNA.

Although, there isn't yet any information available about the diagnostic changes in the cellular elements of the peripheral blood smear as well as Buffy coat preparations of the autistic children, several studies related autism to a decrease in the number of the Purkinje neurons in the cerebellum, Courchesne (1997), who observed a decreased number of Purkinje neurons in recent autopsies and/or quantitative magnetic resonance imaging studies of autistic patients. In addition, he mentioned that since neurogenesis occurs during approximately the 5th week of gestation, the possibility is raised that if an animal model research is exposed to anticonvulsant medication during embryogenesis; it causes a loss of the Purkinje neurons throughout the posterior cerebellum.

It is well known that mitochondria are the energy-producing units of the cells. It is a source of production of Adenosine Triphosphate (ATP), the chemical energy in all living matter derived from oxidative phosphorylation Lombard (1998). He added that the function of the brain is critically dependent on ATP production; oxidative phosphorylation via the mitochondria provides over 95% of total brain ATP. Therefore, he added, adequate mitochondrial metabolism is essential for normal brain function and related the possible cause of mitochondrial toxicity to the production of increased free-radicals of nitric oxide which play a key role in immune-mediated neurotoxicity via mitochondrial inhibition.

CONCLUSION

In conclusion, the present study, described for the first time, comparable cellular and subcellular alterations in blood cells as well as platelets in both autistic candidates and their mothers, the most significant results were noticed among nuclear chromatin, nuclear membrane (including, nuclear blebbing and clefing), as well as cellular organelles specifically mitochondria. The mentioned alterations highlight other converging findings, point toward the mechanism of the production of maternal antibodies with immune reactivity against epitopes of fetal brain and specifically the Purkinje cells as believed by some investigators. This may contribute to the neuro-developmental disorder in autistic candidates.

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