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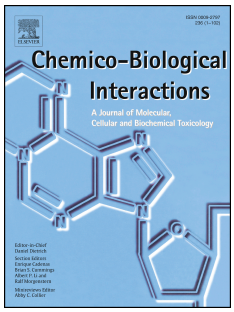

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Erythrocytes as a biological model for screening of xenobiotics toxicity

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Abstract

Erythrocytes are the main cells in circulation. They are devoid of internal membrane structures and easy to be isolated and handled providing a good model for different assays. Red blood cells (RBCs) plasma membrane is a multi-component structure that keeps the cell morphology, elasticity, flexibility and deformability. Alteration of membrane structure upon exposure to xenobiotics could induce various cellular abnormalities and releasing of intracellular components. Therefore the morphological changes and extracellular release of haemoglobin [hemolysis] and increased content of extracellular adenosine triphosphate (ATP) [as signs of membrane stability] could be used to evaluate the cytotoxic effects of various molecules. The nucleated RBCs from birds, fish and amphibians can be used to evaluate genotoxicity of different xenobiotics using comet, DNA fragmentation and micronucleus assays. The RBCs could undergo programmed cell death (eryptosis) in response to injury providing a useful model to analyze some mechanisms of toxicity that could be implicated in apoptosis of nucleated cells. Erythrocytes are vulnerable to peroxidation making it a good biological membrane

model for analyzing the oxidative stress and lipid peroxidation of various xenobiotics. The RBCs contain a large number of enzymatic and non-enzymatic antioxidants. The changes of the RBCs antioxidant capacity could reflect the capability of xenobiotics to generate reactive oxygen species (ROS) resulting in oxidative damage of tissue. These criteria make RBCs a valuable *in vitro* model to evaluate the cytotoxicity of different natural or synthetic and organic or inorganic molecules by cellular damage measures.

Keywords: Erythrocytes, xenobiotic, toxicity, ROS, eryptosis, blood.

1. Introduction

Red blood cells (RBCs) represent the main cells in the body circulatory system, and they act as oxygen transporters. The RBCs of mammals are devoid of nucleus and other cellular organelles while non-mammalian RBCs (birds, fish and amphibians) are nucleated cells however, both types of RBCs have a plasma membrane with specific composition and structure which is highly correlated to their biological functions [1,2].

The main component in the RBCs membrane is protein which represents about 39.5% followed by lipids [35.1%] then water [19.5%] and finally carbohydrates [5.8%] [3]. The structure of RBCs membrane resembles that of other eukaryotic cells. It contains a phospholipids bilayer, integral proteins stabilized in the lipid leaflet by covalent bonds and hydrophobic bonds and a membrane skeleton which is a multi-protein complex composed of some structural proteins including spectrin [α and β], actin, ankyrin and protein 4.1. [4]. The interaction between the membrane components make it elastic and soft and enable its fluctuations and thus provide RBC with elasticity, flexibility and deformability which are very important for RBC to keep its structural integrity and original shape and to protect it from the force of the circulating fluid applied on it during

their passage into the microcirculation [5]. This interaction is metabolically active and need [occur in] the presence of Adenosine Triphosphate [ATP] which is important to protect the cells from fragmentation and the membrane from vesiculation [6, 7]. The cytoskeleton is also very important to keep the cellular components particularly, haemoglobin [Hb] which is the major protein in RBC and it is responsible for transporting oxygen [O_2] and carbon dioxide [CO_2] to and from tissues. Therefore, the level of intra cellular haemoglobin could be used for determining the cytoplasmic viscosity. Meanwhile, the fluidity of membrane lipids depends generally on the type of phospholipids, the acyl chain length, the degree of fatty acid saturation, the presence of free or esterified cholesterol in addition to some amphipathic compounds such as lisophosphatides [8, 9]. Therefore, the changes in the RBCs membrane could be used as indicators for the cell physiological conditions or their possible alterations. The RBCs could be isolated and handled easily so that they could provide a good model for many assays [10, 11]. The application of *in vitro* cytotoxicity assays in RBCs could be used as alternative tools to screen and evaluate the toxicity of different xenobiotic [12]. Additionally, the high concentration of polyunsaturated fatty acids in RBCs membrane, the high oxygen tension, and redox active hemoglobin molecules [the source of reactive oxygen species in RBC] make them a good biological lipid membrane model especially for screening the oxidative stress conditions induced by xenobiotic [13].

Exposure of erythrocytes to oxidative stress lead to lipid peroxidation that could alter the membranes of RBCs inducing membrane protein conformation and protein cross-linking by decreasing membrane protein content and consequently lead to abnormal cell morphology and hemolysis that could disturb the microcirculation [14, 15]. Xenobiotics

including oxidants could trigger the RBCs programmed death which is known as eryptosis and exhaust their antioxidant defense mechanism [16]. Some cysteine proteases-caspases present in RBCs could be also activated after exposure to oxidative stress such as caspase 8 which is a mediator bounded to RBC membrane that could initiate the cellular cascade for apoptosis and caspase 3 which is an effector mediator causing proteolysis of cellular proteins [17]. Energy depletion in the cell beside osmotic shock could also trigger eryptosis [18]. This review is an overview on the different changes that could occur to RBCs in response to different xenobiotics and the role of these alterations in understanding the different mechanisms of xenobiotics toxic actions with an enumeration of some methods for determination of such effects used in previous studies depended on RBCs as a biological model for cytotoxicity assays.

2. Erythrocyte cellular abnormalities

The hematological changes serve as an early indicator for screening the toxic impacts of xenobiotics on tissues [19]. Toxic substances could induce either direct or indirect damage to the RBC cytoskeleton and disturb cell metabolism and ion permeability of RBCs, thus lead to abnormalities in the cellular morphology [20].

The nucleated RBCs from birds, fish and amphibians considered as important models for studying cellular abnormalities. The abnormality in RBCs morphology of such species could be classified in to two main categories: the first one is nuclear abnormalities [ENA] which include: binucleates [BN], lobed nuclei [LB], notched [NT], nuclear bud [NBu] and vacuolated nuclei [VN] according to the identification of Fenech et al. [21]. The second category is cytoplasmic abnormalities [ECAs] such as acanthocytes [AC], echinocytes [EC], vacuolated cytoplasm [VC], notched cytoplasm [NC] and microcytes

[MC] [22]. The shape of RBCs from different animal species and structure of RBCs membrane are represented in Figures (1) and (2).

Most of erythrocytes abnormalities were detected after exposure to toxic substances such as pesticides, metals, chemical agents, irradiation and some types of drugs [8]. Immature pycnotic and mitotic RBCs and with severe anemia were observed in chicken after lead poisoning and poikilocytosis and anisocytosis in swans [23]. Anisocytosis, Poikilocytosis and altered hematocrit and mean corpuscular volume with changes in hemoglobin were also observed in rats exposed to lead [24]. Exposure to lead shot changes nuclear morphology in the blood of domestic fowl (young chickens) [increased RBCs with pycnotic nuclei, enucleated RBCs and reticulocytes] [25]. Exposure of Swiss albino mice to lead during gestation and lactation led to various hematological disorders in RBCs of neonates and abnormal types and sizes [Macrocytosis] and reduced their life span resulting in anemia [26].

Fragmented RBCs [schistocytes] and RBCs with larger sizes were reported after exposure to phenylhydrazine in calf and aluminum in rats [27]. Comelekoglu et al. [28] stated that some pesticides may cause alterations in surface shapes and size of human RBCs. Zeni et al. [29] reported the occurrence of echinocytes in Ictalurus melas after exposure to the anionic detergent [sodium dodecyl benzene sulphonate] as a result of adaptation of the cellular physiological parameters required for shape maintenance. In addition, Koc et al. [30] reported changes in surface shapes and structural defects of RBCs of rats exposed to malathion and endosulfan. Suwalsky et al. [31] have reported that human RBCs when incubated with aqueous extract of *Aristotelia chilensis* showed morphological alterations represented by echinocytic form. Chlorpyrifos provoked

alterations in the cytoskeleton [protein and lipids] of RBCs from Wistar rats thus affecting the cell surface area [32].

3. Characterization of erythrocyte morphology and size

Isolation of RBCs occurs by centrifugation of heparinized blood at 4 ^{O}C for 10 minutes at 3000 rpm, and then buffy coat and plasma were discarded. The erythrocytes were pelleted by centrifugation after being washed once with 0.9% NaCl solution and twice with ice-cold phosphate buffered saline [PBS] [33].

The abnormalities of RBCs morphology could be detected by smearing of pelleted RBCs on glass slides and left to dry then the slides were fixed in absolute methanol for 15 minutes and stained with freshly prepared Giemsa stain and examined under microscope as described by Sharma et al. [26]. The morphological abnormalities of RBCs could be also detected by examining the packed erythrocytes under scanning electron microscope [SEM] after fixation with glutaraldehyde according to method of Agrawal and Sultana [34]. While, alterations in the relative size of RBCs could be determined using flow cytometry using forward scatter [FSC] correlating with cell volume and size [35].

4. Haemolysis, osmotic fragility and protein content in the hemolysate

The *in vitro* haemolytic assay using spectrophotometer represents an effective and easy test for the quantitative measuring of haemolysis [36]. The *in vitro* haemolytic activity test has been reported to be an alternative method in screening for the cytotoxicity of various compounds. It is rapid, reproducible and costless test so it could decrease the use of experimental animals for *in vivo* testing [37]. Many researchers have used the haemolysis assay for evaluating the cytotoxicity of some herbal plants, mushrooms [36,

38] and extracts from different algae [39] to exclude the natural or synthetic products with possible cytotoxic impact which has been prepared for pharmaceutical uses [40].

The changes in osmotic pressure of RBCs could also change their osmotic fragility and cell integrity so it is could be also used as a diagnostic tool in hemolytic conditions [41]. The *in vitro* osmotic fragility of erythrocytes could be determined by the method of Chikezie [42].

The RBCs membrane resistance has been used by many authors as tool in assessment of toxicity for example chlorpyrifos [43], fluoride [44] and 2,4-dichlorophenoxyacetic [45] toxicities in rat RBCs. The haemolytic assay is based on measuring the release of haemoglobin from RBCs suspended in solution with gradual reducing the concentration and detecting the cells that showed osmotic lysis [concentration-response]. The concentration of released hemoglobin [protein] is correlated to the percentage of lysed cells. Hemoglobin and protein contents in the hemolysate could be determined photometrically at 540 nm. The absorption of the hemolysate of RBCs lysed in distilled H_2O was defined as being 100% haemolysis. The percentage of haemolysis could be calculated by the following equation:

$$Haemolysis(\%): \frac{\mathrm{Abs_{sample}}}{\mathrm{Abs_{erythrocytes\ in\ water}}} \times 100$$

5. Changes in cellular energy [ATP]

The ATP is used by RBCs to maintain osmotic stability and keep submembrane skeletal-network proteins thereby maintain membrane shape and control deformation [46]. Meyers and Hendricks [47] reported that cellular injuries decreased the oxidative phosphorylation process inside the cell resulting in reduced ATP content which consequently forces cells to

show vacuolization and lead also to unequal distributed hemoglobin which resulted in RBCs cytoplasmic vacuoles [48]. Exposure of rat RBCs to lead [Pb] shortened their lifespan due to inhibition of the Na-K-ATPase and loss of membrane integrity [49]. A more recent study on cyadox suggested its role in induction of energy depletion in isolated rabbit RBCs by decreasing their ATP contents [50]. Sikora et al. [51] stated that the only source of extracellular ATP is cell lysis. So determination of ATP content of RBCs can give idea about the status of their membrane integrity and energy charge as well.

Measurement of the ATP and determination of erythrocytes energy charge: The measurement of the ATP content could be performed according to the method developed by Adams [52] with ATP expressed as µmol/g Hb or through measuring the intracellular ATP content using luciferin–luciferase assay kit. To determine the adenylates contents, ADP and AMP were measured as the difference after their enzymatic conversion to ATP [53]. The adenylate energy charge [EC] was calculated by the equation; EC = [[ATP] + 1/2[ADP]]/[[ATP] + [ADP] + [AMP]] [54].

6. Erythrocytes as a tool in genotoxicity assays

Screening for genotoxic impact is of great importance during the evaluation of xenobiotic cytotoxicity as the genotoxic potential is usually implicated in carcinogenic and reproductive toxicities as a primary risk factor therefore; genotoxicity testing are helpful for describing the ability of different xenobiotics to damage the cellular genetic information and to induce mutation. For analyzing the effect of a genotoxic molecule, DNA damage in cells exposed should be evaluated. The DNA damage can be in the form of single-strand, double-strand breaks, cross-linking, loss of excision repair, point

mutations, alkali-labile sites and chromosomal aberrations [structural and numerical] [55].

Genotoxic effect of xenobiotics on isolated RBCs could be evaluated using different assays including micronucleus assay, comet assay and DNA fragmentation assay as follow:

The micronucleus assay [MN]: The MN is an easy test used as a marker of genotoxic effect of different pollutants in bio-monitoring studies for assessing of their cytogenotoxic potential in field and in laboratory conditions. The appearance of micronucleus in a cell indicated the occurrence of chromosomal aberrations during mitosis [numerical or structural] [21]. The MN assay was performed earlier on the mammalian RBCs [especially rodents], then it has been widely used along with other cellular abnormalities [ENA and ECA] as endpoints in several species of fish and other aquatic organisms [56] and also in different species of birds [57] exposed to various toxicants. Tan et al. [58] reported that there is a positive correlation between the frequencies of MN and other nuclear abnormalities. The MN test was helpful in evaluating genotoxicity of $CdCl_2$ in gill cells and haemocytes of *Dreissena polymorpha* [59]. The MN was observed in RBCs of chickens exposed to endosulfan [57]. To detect micronuclei in erythrocytes, few drops of whole blood were directly smeared on clean slides. The slides were left for 24 h to dry, fixed for 10 min in methanol, stained by 10% Giemsa , then analyzed using a 1000· oil-immersion lens [60].

The comet assay: The comet assay is rapid, potent and economic test for studying DNA damage induced by chemical and physical agents [61]. This assay is sensitive in detecting DNA damage even if the level of damage is low, and could be performed on

small number of cells and could generate data at individual cell level. It has been widely employed in environmental cytogenotoxicity monitoring in a variety of fish species. Comet assay in RBCs was helpful in detecting cadmium chloride induced genotoxicity and cytotoxicity in freshwater fish *Labeo rohita* [35].

DNA fragmentation assay: The DNA fragmentation of the RBCs could be isolated according to the method developed by Weil et al. [62]. Then the fragmented DNA was analyzed by agarose gel electrophoresis at 40 V for 5 h using agarose gel [1%] then examined and photographed as described by Wang et al. [63].

7. Erythrocyte programmed death [Eryptosis]

The death of RBCs caused by injury is a more complex process than haemolysis and it resembles the apoptosis [suicidal death of nucleated cells] which is important for the disposal of deformed cells without rupturing the cellular membranes [18]. Apoptosis of nucleated cells has been used for evaluating the toxic effects of various xenobiotics that could be implicated in disrupting the cell function and tissue destruction [64]. Apoptosis occurs through a chain of events, including nuclear condensation and shrinkage, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing and exposure of phosphatidyl serine on the plasma membrane [breakdown of phosphatidylserine asymmetry of the plasma membrane] [65].

Several researches showed that mammalian RBCs however lack nuclei they have the ability to undergo a programmed death [eryptosis] upon exposure to injurious materials and endogenous challenges which affect the cell integrity and arrest their life cycle [39, 66]. Therefore, eryptosis could be used as a model for analyzing different mechanisms that are of the same importance for the apoptosis of nucleated cells. Simpson and Kling

[67] reported that erythroblasts of dogs exposed to phenylhydrazine showed denucleation without condensation of chromatin. The RBCs of embryonic and newly hatched chickens exposed to cycloheximide or staurosporine showed DNA fragmentation and pycnosis [62]. The RBCs of human respond to lead by surface exposure of phosphatidylserine and shrinkage of cells [68].

During apoptosis, caspases function either as initiators such as caspase-8 and -9 (the membrane-bound mediator initiates the cellular cascade for apoptosis) in response to proapoptotic signals or as effectors such as caspase-3 (the effector mediator leading to cellular proteins proteolysis) . Mature erythrocytes contain considerable amounts of caspase-3 and caspase-8 whereas other essential components of the mitochondrial apoptotic cascade such as caspase-9, Apaf-1 and cytochrome c are absent [69]. Eryptosis could be triggered by osmotic shock and energy depletion in RBCs [17]. Generation of ROS, depletion of antioxidants and formation of adducts are important factors promote death process and lead to the cell disintegration [70]. Chronic arsenic exposure led to ROS generation in the RBCs of rats and consequently led to the activation of caspase 3 [71]. The significant increase in caspase 3 and 8 were also observed in rabbit erythrocytes exposed to cyadox [50].

Some toxic substances can lead to formation of ceramid in RBCs of some animals like rat and mice [44, 72]. This could be returned to the activation of Gardos channel. The Garods channel activation induces the shrinkage of RBCs and consequently activates the sphingomyelinase [aSMase] and enhance more ceramid generation which triggers suicidal erythrocyte death [73]. Ceramid formation has been also reported to help the super aggregation [capping] of Fas receptors [translocated to lipid raft of RBCs

membrane] and are highly essential for formation of death inducing signaling complex [DISC] and other downstream events associated with Fas induced apoptosis [74]. So, determination of caspase activities, ceramid formation, Garods channel activation, aSMase activity and Fas aggregation could be used to evaluate the apoptotic effects of xenobiotics on RBCs.

Determining the lifespan of RBCs: Survival of RBC could be measured from the half-life of erythrocyte over time according to the standard method of Sen et al. [75].

Determination of ceramide formation: Ceramide contents in RBCs could be determined by antibody based fluorimetric method [76].

Determination of Gardos channel activity: Gardos channel activity could be determined by the method of Wolff et al. [77].

Determination of aSMase activity in RBCs: aSMase activity could be quantified according to the method of Petrache et al. [78].

Fas aggregation on erythrocyte membrane: For determination of Fas aggregation, the lipid raft should be firstly isolated and this could be done by the method of discontinuous density gradient ultracentrifugation according to Muppidi and Siegel [79], then Fas aggregation [translocation of Fas to lipid raft] could be determined by immunohistochemistry with Fas antibody [74].

Determination of caspase activities: The proteolytic activity of caspase8 and caspase 3 could be measured in RBCs lysate as described in Mukherjee et al. [72].

8. Erythrocytes as an initial screen for oxidative stress

Erythrocytes have been extensively used as a biological membrane model to analyse the oxidative damage as they are highly vulnerable to peroxidation owing to the high content of polyunsaturated fatty acid [PUFA] in their membrane, their role as O_2 and CO_2 transporters and the presence of redox active hemoglobin molecule , which is a potent source of reactive oxygen species [ROS]. They also contain heme-iron which is ferrous hemoglobin essential for the hydrogen peroxide-stimulated oxidation of lipids in the RBCs membrane [13, 80]. Redox regulation in RBCs is shown in Figure (3).

Oxidative damage to RBCs after exposure to xenobiotics [chemicals, drugs, metals, pesticides, and irradiations] induced alterations in morphology of cells, membrane protein conformation, protein cross-linking, lipid peroxidation and consequently hemolysis of RBCs [14, 15]. Oxyhemoglobin and autoxidation are the main sources of intracellular ROS in the RBCs [81]. Oxidative stress impairs oxygen delivery and induces RBCs aging [82]. Different effects of xenobiotics on erythrocytes are shown in Figure (4).

Erythrocytes are protected against oxidative damage by various biological mechanisms including antioxidant enzymes as superoxide dismutase [SOD] and catalase [CAT] [83]. SOD and CAT protect cells by scavenging the free radicals and ROS elimination. SOD converts the highly reactive superoxide anion to a less reactive species, H_2O_2 and to O_2. SOD contains zinc to keep its stability and copper to maintain its activity [84]. Concerning CAT, more than 98% of blood CAT is present in the RBCs and it is able to remove extra and intra-cellular H_2O_2 giving protecting for tissues deficient in CAT activity [85]. Therefore, blood CAT activity could be used as a good bioindicator for the overall protection against the diffusible H_2O_2.

Reduced glutathione [GSH] is a non-enzymatic antioxidant that protects lipids and proteins in RBCs membrane and keeps its stability, enhances the RBCs survival against oxidative injury and provides the primary antioxidant defense for the stored RBCs [86]. GSH protects important proteins in RBCs against oxidation and acts as vital sulfhydryl buffer, which keeps SH groups in enzymes and Hb in the reduced state [84]. Therefore, GSH depletion could increase oxidative stress and modify the RBCs components.

4-Hydroxynonenal [4-HNE], is one of the major alpha beta unsaturated aldehydes formed in erythrocytes as a result of peroxidation of membrane lipids after oxidative injury. 4-HNE induced reduction of intracellular GSH by forming GSH-HNE adducts in cytosol and interacted with cytosolic and membrane proteins forming HNE–protein adducts. These adducts resulted in depletion and modulation of antioxidant activities, increasing production of ROS and disruption of RBCs redox status [87]. HNE–protein adducts formations were observed in erythrocytes of rats after exposure to chronic arsenic toxicity [71]. While GSH-HNE adducts were used as a tool to detect lead toxicity on mice RBCs by Mukherjee et al. [72].

Lipid peroxidation [LPO] is one of the consequences of oxidative damage, and it is one of the chief mechanism for cell injury and death [i.e., hemolysis] [84]. Malondialdehyde [MDA], the well-characterized product of the LPO of RBCs, is a highly reactive bifunctional molecule, that could impair various membrane functions by cross-linking the RBCs proteins and phospholipids leading to diminished survival and death [induce hemolysis] [88, 89]. Moreover, LPO of RBCs may be implicated in cell aging, and variable pathological conditions. The determination of MDA level provides a good measure of LPO [90].

Additionally, erythrocytes SOD contains copper and zinc [CuZn-SOD]. The interaction between CuZn-SOD and MDA modified the residues of histidine amino acid and produced protein–protein cross-linked derivatives therefore each type of ROS exhibits a different pattern of protein oxidation. So the protein carbonyl content could be used as an indicator of protein oxidation in erythrocytes as reported by Reddy et al. [91].

Erythrocytes were proved to be a good tool for analyzing the oxidative stress and lipid peroxidation as mechanism of toxic action in various studies. RBCs help in assessing the toxicity of arsenic [71] and 2, 4-dichlorophenoxyacetic [45] in rats; lead toxicity in mice [72] and Lambda-cyhalothrin [92] and cyadox [50] in rabbits.

Some toxic substances could induce pronounced changes in the fatty acid profiles of RBCs membrane due to free radical generation which transferred the both types of membrane fatty acids [saturated and unsaturated] from superficial neutral lipids into phosphatidylethanolamine. For example 2,4-dichlorophenoxyacetic induced cellular deterioration through peroxidation of PUFA ,degradation of membrane phospholipids and increasing the index of fatty acid unsaturation [SFA to UFA ratio] [45]. Alterations of fatty acid composition of RBCs membrane could result in changing the membrane viscosity [93]. Exposure of erythrocytes to ROS generated during arsenic toxicity could result in alterations of membrane proteins [71].

A- Oxidative stress parameters

Preparation of RBCs membrane [erythrocyte ghosts]: erythrocyte ghost could be prepared by the method of Dodge et al. [94].

Fatty acid composition of erythrocytes: The fatty acid composition of erythrocyte was analyzed by gas chromatography after trans-esterification following the method of Giacometti et al. [95]

Electrophoresis of membrane proteins: polyacrylamide gel electrophoresis with dodecyl sulphate [SDS-PAGE] of RBCs membrane proteins could be determined according to Laemmli [96].

Assessment of lipid hydroperoxide content in RBCs membrane: could be estimated using the FOX2 method [97].

Determination of RBCs redox potential: Redox potential in RBC could be determined by the protocol of Biswas et al. [98] from the ratios of pyridine nucleotides. Redox potential was assayed from NADH/[NAD++ NADH] and NADPH/[NADP++NADPH] ratios.

Estimation of intracellular reactive oxygen species [ROS]: The ROS content of RBCs cold be measured by incubating with fluorescent probes dihydroethidium [DHE] and 2′,7′-dichlorofluorescein diacetate [DCFDA] according to Lopez-Revuelta et al. [99]; and Zhao et al. [100], then fluorescent-positive cells were detected by flow cytometer according to [101].

The H_2O_2 and $O_2^{-\bullet}$ concentrations of RBCs: could be determined by the method of Qian et al. [102]. While sulfhydryl group [SH] groups could be measured in RBCs after reaction with 5, 50-dithiobis-[2-nitrobenzoic acid] using the method of Ellman [103].

Measurement of lipid peroxidation of erythrocytes: There are many different methods that could be used for measuring the lipid peroxidation in RBCs. Malondialdehyde

[MDA], a product of lipid peroxidation was measured at 532 nm by using 2-thiobarbituric acid [2, 6-dihydroxypyrimidine-2-thiol; TBA] [104] where MDA concentration was expressed either as nmol/mg protein for the membranes or nmol/gHb for whole erythrocytes. Lipid peroxidation could be assessed indirectly through measurement of the thiobarbituric acid [TBA] reaction [105] where MDA values were expressed as pmol/g Hb. MDA levels could be also measured by the method of Bartosz [106] using the thiobarbituric acid [TBA] method, after the reaction with TBA, the reaction product was measured spectrophotometrically at 535mm where the MDA level was expressed in M/gHb. The method of Tedesco et al. [107] could be also used to measure the level of Lipid peroxidation in RBCs in terms of MDA equivalents using the thiobarbituric acid reaction.

Protein oxidation: Protein carbonyl content is used as a marker of protein oxidation in RBCs and it could be measured using the method described by Uchida and Stadtman[108] or according to Jiang et al109] .]

Measurement of GSH–HNE and HNE–protein adduct formation: GSH–HNE adduct could be measured using Mass spectrometry as described by Biswas et al. [71]. While HNE–protein adduct formation could by analyzed according to Arguelles et al. [110].

B- Determination of erythrocyte antioxidants

Reduced glutathione [GSH] and oxidized glutathione [GSSG] estimation: GSH content in RBCs could be detected by different methods. It could be measured at 25 °C according to the standard methods using DTNB as described by Tietze [111] where GSH content was expressed as lmol/g Hb or by the method of Dise and Goodman [112]

where GSH was expressed as nmol GSH per mg of hemoglobin. While, intra-cellular GSSG content could be measured at 340 nm as described by Beutler [113].

Glutathione peroxidase [GSH-Px],Glutathione-s-transferase [GST] and glutathione reductase [GR] activities: The GSH-Px could be evaluated using Ransel reagents based on the method of Paglia and Valentine [114]. Results were expressed in U/g Hgb. While GST activity was measured spectrophotometrically by the method of Habig et al. [115] using S-2,4-dinitro phenyl glutathione [CDNB] as a substrate. The activity of GST was expressed in terms of nmol/mg protein. The glutathione reductase GR activity was assayed using reagent from Randox Laboratories [GR 2368]; the assay was adapted from the method of Beutler [113]. Results were expressed in U/g Hgb.

SOD and CAT activity: The SOD activity could be determined according to the method of Marklund and Marklund [116] by measuring the inhibition of pyrogallol autoxidation. One unit of SOD was defined as the amount of enzyme that inhibits the rate of pyrogallol autoxidation in 50%. The SOD activity in RBCs could be also determined by using the classical NBT method [117] where SOD activity expressed as U/mg Hb. while CAT activity in erythrocyte lysate could be determined according to the method of Aebi [118]. The method is based on the decomposition of H_2O_2 by catalase. Enzyme activity was expressed as units per mg of Hb [U/mg Hb].

Types of damage resulted from interaction of some xenobiotics and RBCs from different animal species are illustrated in Table 1.

Conclusion

This review shows that erythrocytes could be considered as a valuable model in studying the cytotoxic effect of xenobiotics owing to its particular structure and the important components of their plasma membrane especially lipids and proteins. The disturbance of plasma membrane integrity leads haemoglobin to be released extracellular so the hemolytic activity of RBCs could provide useful information about the interaction of different molecules and the biological activities on the cell level and could be used to exclude the pharmaceutical products with possible cytotoxic effects. The RBCs membrane could be altered and deformed in response to injurious xenobiotics resulting in cellular abnormalities in morphology and size which could be used as initial testing for cytotoxicity. ATP content of RBCs can give idea about the status of their membrane integrity and energy charge as the only source of extracellular ATP is lysis of cells. Exposure of the body to different physical and chemical agents can result in genotoxic and DNA damaging effects and lead to carcinogenic and reproductive toxicities. RBCs can be used for evaluating the genotoxic impacts of different xenobiotics using different assays including micronucleus assay, comet assay and DNA fragmentation assay.

The RBCs however lack nuclei they have the ability to undergo a programmed death [eryptosis] upon exposure to injurious materials and endogenous challenges. Therefore, eryptosis could be used as a model for analyzing different mechanisms that are of the same importance for the apoptosis of nucleated cells. Erythrocytes were proved to be a good biological membrane model for analyzing the oxidative stress and lipid peroxidation as possible mechanisms of toxic action of various xenobotics because of their vulnerability to peroxidation as RBCs contain polyunsaturated fatty acid [PUFA] in their membrane and hemoglobin which is a source of ROS and due to its capacity to

transport O_2 and CO_2. Oxidative damage could alter the membrane lipid and protein structure and cellular morphology and induce lipid peroxidation and hemolysis therefore; lipid peroxidation could be also of value in understanding the mechanism of action of various xenobiotics. The RBCs are well equipped by various biological mechanisms of antioxidants including enzymes and non-enzymatic antioxidants which also could be altered by oxidative damage. The changes of component of RBCs, the structure of plasma membrane and the antioxidant capacity of RBCs could be used as good indicators of the ROS generating activity of xenobiotics and their oxidative damaging effects on tissues. All these findings make the RBCs a valuable in vitro initial screening model for the evaluation of cytotoxic mechanisms of various substances and prove their ability to be used as alternative testing methods to decrease animal experimentation, helping reach the goal of reducing, refining and replacing studies conducted with animals which requires major ethical and financial regulations.

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Competing interests:

Authors declare that there are no competing interests.

Author contributions:

MRF and MA wrote, revised and reviewed the manuscript.

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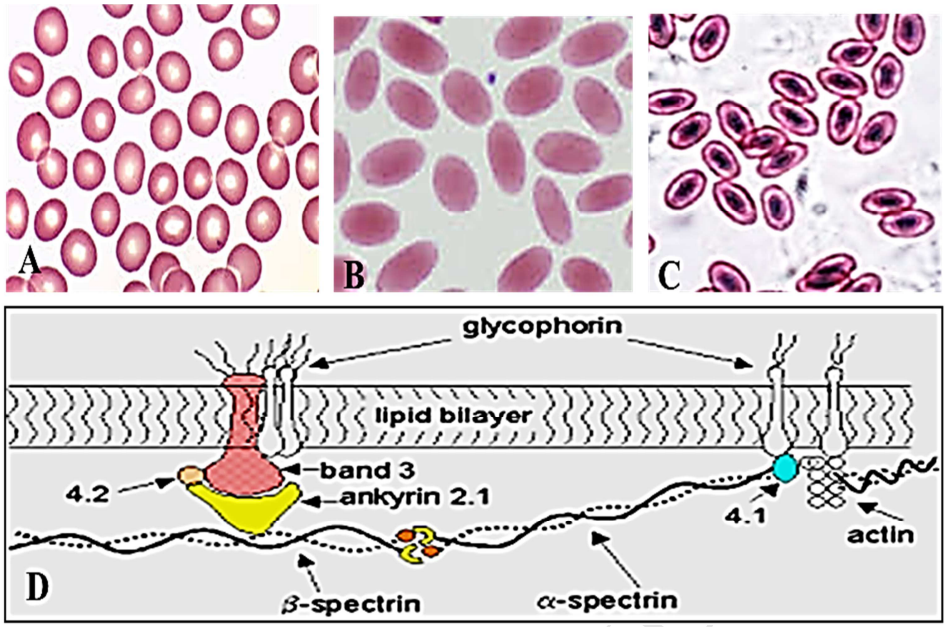

Figure 1. A, erythrocytes of mammals (rounded, non-nucleated). B, erythrocytes of camel (oval, non-nucleated). C, erythrocytes of birds, fish and amphibians (oval, nucleated). D, the structure of erythrocyte membrane (Figures are collected from the internet)

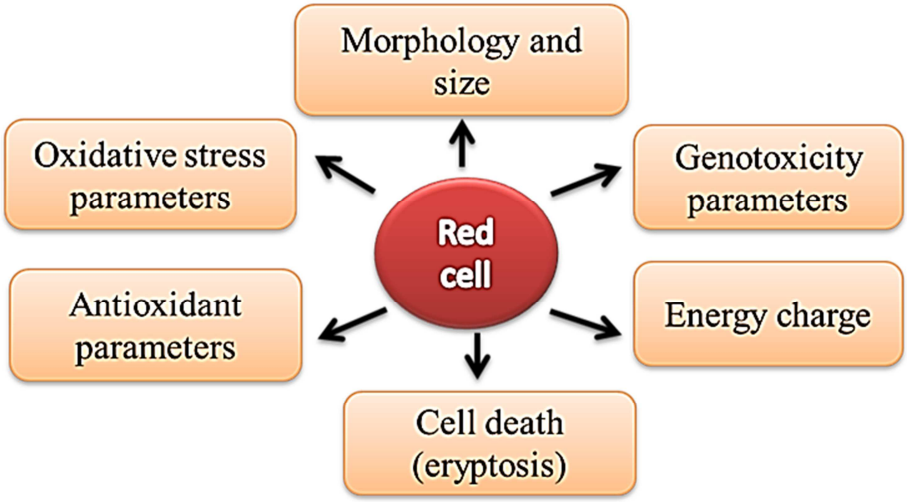

Figure2. Erythrocytes as a model for evaluating the cytogenotoixc effects of xenobiotics

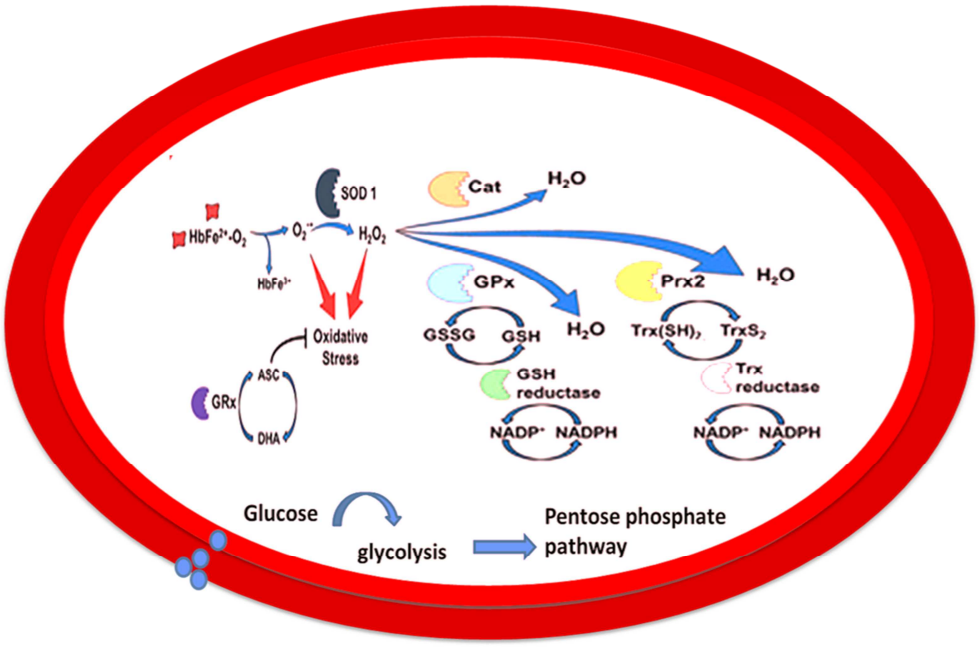

Figure 3. Redox regulation in RBCs

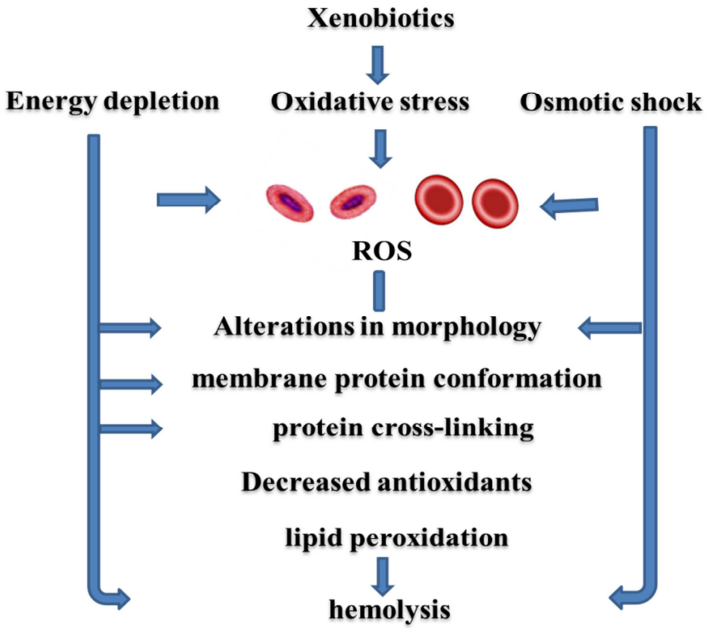

Figure 4. Different effects of xenobiotics on erythrocytes

Table 1. Type of damage resulted from interaction of some xenobiotics and RBCs from different animal species. 987 988

The xenobiotic	The type of damage to RBC	The species of the RBC	References
Lead	Oxidative stress , apoptotic events (Fas aggregation in lipid rafts led to Fas-dependant death of RBCs) GSH and GSH–HNE adduct Gardos channel Ceramid formation inhibition of acid sphingomyelinase (aSMase)	Mice	[17]
	poikilocytosis and anisocytosis	Whooper swans (*Cygnus Cygnus*)	[23]
	Anisocytosis, Poikilocytosis ,changes in hemoglobin and oxidative damage	Rats	[24]
	Pycnosis and enucleation of peripheral erythrocytes	Chicken	[25]
	shortened lifespan , inhibited Na-K-ATPase and loss of membrane integrity	Rat	[49]
	Stimulation of phosphatidylserine exposure at the erythrocyte surface	Human	[68]
	Fas induced apoptotic events in erythrocytes	Mice	[72]
	Abnormalities of RBCs	Chickens	[119]
	genotoxicity (micronuclei and other nuclear abnormalities	Freshwater fish Prochilodus lineatus	[120]
	oxidative damage	Rat	[121]
	Decreased antioxidant levels	Goats	[122]
	Morphological changes	Human	[123]
	Altered activity of potassium-selective channels	Human	[124]
	Induction of micronucleus	The fish H. Malabaricus	[125]
Copper	Genotoxicity (increased micronuclei and other nuclear abnormalities)	Freshwater fish species viz. *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella*	[126]
Cadmium chloride and copper sulphate	micronuclei and binuclei	Fish Common carp (*Cyprinus carpio*), Prussian carp (*Carassius gibelio*)	[127]

		and Peppered cory(*Corydoras paleatus*)	
Cadmium chloride	genotoxicity and cytotoxicity cellular abnormalities and DNA damage	Fresh water fish Labeo rohita (Hamilton)	[35]
	Genotoxic effects	*Dreissena polymorpha*	[59]
Cadmium, copper, lead and zinc	Genotoxicity and cytotoxicity	Nile tilapia, (*Oreochromis niloticus*)	[128]
Mercury	Induction of micronuclei	Cyprinus carpio	[129]
	Nuclear abnormalities	Wild and caged fish (*Liza aurata*)	[130]
Potassium dichromat	hemolytic effect	Rats	131
Arsenic	Fas-activated erythrocyte apoptosis	Rats	[132]
	Reduced cellular redox status, induced 4- hydroxynonenal-mediated caspase 3 activation, erythrocyte death	Rats	[71]
	erythrocyte death	Human	[98]
Aluminium	Morphologic and functional alterations		[27]
	Alter Morphology	Rabbit	[133]
Vanadium aluminium	Oxidative stress	Human	[134]
Iron	oxidative stress	Human	[135]
Fluoride	Apoptosis, altered membrane integrity, altered cell morphology and size, induced moderate ceramide formation,	Rat	[44]
	Oxidative stress	Mice	[136]
Vanadate and magnesium	Attenuated defence system	Wistar rats	[137]
Iodoacetate, vanadate and ferri cyanide	Peroxidative membrane damage	human	[138]
Lambda-cyhalothrin	Genotoxicity (micronucleus)	Fish (Garra rufa (Pisces: Cyprinidae)	[139]
	Oxidative stress	Rat	[140]
	Oxidative stress decrease the activity of	Rabbit	[92]

	acetylcholinesterase (AChE)		
	Micronuclei and nuclear abnormalities	Mosquito fish (*Gambusia affinis*)	[141]
	Micronucleus	Erythrocytes of the fish (*Cheirodon interruptus*)	[142]
Organophosphate pesticides (chlorpyrifos and malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin)	Genotoxicity (micronuclei)	Fish (*Oreochromis mossambicus*)	[143]
Pyrethroid	Lipid peroxidative damage and alterations in antioxidant status	Rat	[144]
Phenoxyherbicides	Production of free radicals	Human	[145]
Phenoxyherbicides (e.g., 2,4-dichlorophenoxyacetic acid (2,4-D-Na), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T-Na) and 4-chloro-2-methylphenoxyacetic acid (MCPA-Na)	Altered erythrocyte membrane fluidity and changed membrane proteins content	Erythrocytes	[146]
2,4-dichlorophenoxyacetic herbicide	Attenuated antioxidant defense system and induced lipid peroxidation	Rat	[45]
2,4-dichlorophenoxyacetic acid and butachlor.	Induced micronuclei and erythrocyte alterations	Catfish (*Clarias batrachus*)	[147]
-2,4-dichlorophenoxy acetic-acid- and butachlor	DNA damage by alkaline single cell gel electrophoresis	Catfish (*Clarias batrachus*)	[148]
Chlorpyrifos	Alterations in the profile of blood cells, alterations in the cytoskeleton [protein and lipids] of RBCs from wistar rats thus affecting the cell surface area	Wistar rats	[32]
	Lipid peroxidation and oxidative stress	Rat	[43]
	Genotoxicity, micronucleus	Freshwater fish (*Channa punctatus*)	[149]
Lindane	Oxidative stress	Rat	[150]
hexachlorocyclohexane	Biochemical and structural	Rat	[34]

	alterations		
Fenvalerate and its metabolite.	Morphological and biochemical perturbations	Rat	[151]
Endosulfan	The MN was observed in RBCs of chickens exposed to endosulfan	Chicks	[57]
Malathion and endosulfan	Changes in surface shapes and structural defects of RBCs of exposed to malathion and endosulfan	Rats	[30]
The herbicide (Roundup)	Micronuclei, nuclear abnormalities and DNA damage	Freshwater goldfish (*Carassius auratus*)	[152]
The herbicides AAtrex Nine-O-, Dual-960E-, Roundup-, and Sencor-500F	DNA damage by comet (DNA fragmentation)	Erythrocytes from *Rana catesbeiana* (bullfrog) tadpoles	[153]
The fungicide (Propiconazole)	Genotoxicity (micronuclei)	Fresh water fish (*Clarias batrachus*)	[154]
Tributyltin	genotoxicity (micronucleus)	The fish *H. Malabaricus*	[125]
Anionic detergent	Echinocytosis	Bullhead, *Ictalurus melas Rafinesque*	[9]
Textile mill effluent	Micronuclei and other nuclear abnormalities	*Oreochromis niloticus*	[155]
Petroleum refinery and chromium processing plant effluents	Micronuclei and nuclear abnormalities	*Oreochromis niloticus*	[156]
Waters affected by refinery effluent	Micronucleus test and observation of nuclear alterations	Nile tilapia	[157]
Mitomycin-C (cytotoxic antineoplastic agent)	Nuclear abnormalities (micronuclei and nuclear buds)	Parrots (*Aratinga canicularis*)	[158]
Mitomycin C and cyclophosphamide	Micronuclei	Fathead minnow (Pimephales promelas)	[159]
Cyclophosphamide	Genotoxicity using the micronucleus assay and nuclear abnormalities	The tropical sea fish (*Bathygobius soporator*)	[160]
5-Fuorouracil	Micronuclei	Mouse	[161]
Cyadox	Altered antioxidant defense system and induced hemolysis	Rabbit	[11]

	Induced energy depletion by decreasing their ATP contents	Rabbit	[50]
2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) and 4-amino-3,3'-dichloro-5,4'-dinitro-biphenyl (ADDB)	Genotoxicity DNA damage	Goldfish (*Carassius auratus*)	[162]
Dialkyl phthalate, bisphenol A, tetrabromodiphenyl ether	Micronuclei	Fish (*Scophthalmus maximus*)	[163]
Tert-Butyl hydroperoxide	Alters fatty acid incorporation into erythrocyte membrane phospholipid.	Human	[112]
4-nonylphenol	ATP content which consequently forces cells to show vacuolization and lead also to unequal distributed hemoglobin which resulted in RBCs cytoplasmic vacuoles	African catfish (*Clarias gariepinus Burchell*)	[48]
acrylamide	Cytogenetic effects	*Carassius auratus*	[58]
Naphthalene and β-naphthoflavone	Erythrocytic nuclear abnormalities	*Anguilla anguilla* L.	[164]
Ethyl methane sulphonate	Nuclear anomalies (micronuclei)	Fish (eastern mudminnow Umbra pygmaea	[165]
Ethanol	erythrocyte membrane	Rats	[166]
Hydrogen peroxide	Oxidative damage	Rat	[167]
Irradiation (gamma-ray)	Induced biochemical changes, generate reactive oxygen species (ROS), deformability, lipid peroxidation	Human	[168]
X-rays and colchicines	Micronuclei induced	*Cyprinus carpio* (teleostei, pisces)	[169]
Flavonoids of Aristotelia chilensis Maqui leaves	Morphological alterations represented by echinocytic form	Human	[31]

Agaricus sylvaticus	Hemolytic activity	Human	[37]
Thymoquinone and limonene	Alleviation of plasma, erythrocyte and liver lipidemic-oxidative stress	Rats	[80]

Highlights

- Exposure of the body to xenobiotics results in cytotoxicity and genotoxicity
- Erythrocytes contain specific structure and antioxidant system
- Erythrocytes with its structure are valuable in screening of xenobiotics toxicity