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Aneta Wolska

Wpływ bisfenolu A i jego wybranych analogów na erytrocyty człowieka

The effect of bisphenol A and its selected analogs on human erythrocytes

Praca doktorska

wykonana w Katedrze Biofizyki Skażeń Środowiska
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pod kierunkiem
dr. hab. Jaromira Michałowicza, prof. nadzw. UŁ

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SPIS TREŚCI:

1. SPIS PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Maćczak A., Bukowska B., Michałowicz J. *Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes.* Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 2015, 176-177: 62-70.

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2. OMÓWIENIE CELU NAUKOWEGO I UZYSKANYCH WYNIKÓW

WPROWADZENIE

Bisfenol A (BPA; 2,2-bis-(4-hydroksyfenylo)propan) jest substancją chemiczną, powszechnie stosowaną jako podstawowy związek do produkcji żywic epoksydowych, żywic fenolowych, poliwęglanów, poliakrylanów, poliestrów i papieru termicznego [Michałowicz 2014; Song i in. 2014; Goodman i in. 2017]. Ze względu na szerokie wykorzystanie, BPA jest obecny w wielu przedmiotach codziennego użytku, takich jak plastikowe butelki i pojemniki do przechowywania żywności, puszki, zabawki dla dzieci, banknoty, paragony, a także sprzęt elektroniczny i medyczny (uszczelniacze dentystyczne, implanty) [Huang i in. 2012; Michałowicz 2014]. BPA jest jedną z substancji chemicznych wytwarzanych w największych ilościach na świecie. W 2003 r. wyprodukowano ponad 3 mln ton tego związku, podczas gdy w 2013 r. globalna produkcja BPA osiągnęła aż 6,8 mln ton [Vandenberg i in. 2007; GrandViewResearch 2014].

Głównym źródłem ogólnopopulacyjnego narażenia na bisfenole jest żywność i woda pitna. Wskutek niekompletnej polimeryzacji materiałów syntetycznych, pozostałości BPA mogą migrować z plastikowych opakowań oraz lakierów (żywic epoksydowych) wyścielających puszki do produktów żywnościowych [Liao i Kannan 2013]. BPA oznaczono również w wodzie pitnej przechowywanej w poliwęglanowych butelkach [Huang i in. 2012; Honeycutt i in. 2017]. Badania dowodzą, że podwyższona temperatura, kwasowe lub zasadowe środowisko roztworów oraz uszkodzenia mechaniczne opakowań wzmagają uwalnianie BPA z polimerów [Vandenberg i in. 2012]. Według szacunków Unii Europejskiej, dzienne narażenie osoby dorosłej na BPA poprzez dietę zawiera się w zakresie od 0,02 do 59 μg/kg masy ciała/dobę [Huang i in. 2012].

Ekspozycja na bisfenole zachodzi także drogą oddechową - wdychanie powietrza zanieczyszczonego tymi substancjami. BPA oznaczono w powietrzu atmosferycznym, pomieszczeniach mieszkalnych oraz w zakładach produkujących BPA i żywice epoksydowe [Corrales i in. 2015]. Badania wykazały także, że BPA obecny jest w kurzu, do którego przedostaje się w wyniku codziennego użytkowania przedmiotów wykonanych z tworzyw sztucznych. Geens i in. [2009] wykazali, że średnia zawartość BPA w kurzu pomieszczeń mieszkalnych i biurowych wynosi odpowiednio 1460 ng/g i 6530 ng/g.

Istotną drogą przenikania bisfenoli do organizmu człowieka są powłoki skórne. Kontakt skóry z papierem termicznym (paragony, bilety, gazety) zawierającym BPA i bisfenol S (BPS) stanowi znaczące zagrożenie zdrowotne dla populacji ogólnej [Geens i in. 2012; Björnsdotter i in. 2017]. Uważa się także, że ważnym źródłem narażenia na bisfenole jest odzież i produkty do pielęgnacji ciała. Związek ten oznaczono w odzieży dla niemowląt oraz kosmetykach (np. szamponach, maseczkach, pomadkach, kremach do opalania) [Lu i in. 2017; Xue i in. 2017]. Narażenie pracowników na bisfenole wiąże się głównie z produkcją tych związków, syntezą żywic epoksydowych i poliwęglanów (kontakt przez skórę i drogi oddechowe), jednak dotyczy również kasjerów mających codzienny kontakt z dużą liczbą paragonów fiskalnych [Michałowicz 2014; Ndaw i in. 2016].

Odzwierciedleniem narażenia populacji ogólnej na bisfenole jest obecność tych związków w tkankach i płynach ustrojowych człowieka. W przeprowadzonym badaniu wykazano obecność BPA w moczu mieszkańców Ameryki Północnej w zakresie stężeń od 0,4 do 149 μg/ml [Calafat i in. 2008]. BPA oznaczono także w zakresie stężeń od 3,3 do 30 μg/ml w ślinie osób posiadających wypełnienia dentystyczne [Vandenberg i in. 2007]. BPA, z uwagi na charakter lipofilny, ulega akumulacji w tkance tłuszczowej w średnim stężeniu wynoszącym 3,16 μg/kg [Fernandez i in. 2007]. BPA w zakresie stężeń od 27,16 do 41,61 μg/l oznaczono w surowicy krwi osób zawodowo narażonych na ten związek [Huang i in. 2012]. Wyniki badań wykazały, że BPA stanowi zagrożenie dla dzieci i płodu. Wskutek ekspozycji prenatalnej (poprzez kontakt matki z BPA), związek ten przenika do tkanki łożyskowej, płynu owodniowego, krwi pępowinowej oraz krwi płodu [Vandenberg i in. 2007; Vandenberg i in. 2012]. Ponadto, BPA oznaczono w mleku matek w średnim stężeniu 0,74 ng/ml [Lee i in. 2017].

Badania toksykologiczne wykazały toksyczne, endokrynne i potencjalnie kancerogenne oddziaływanie BPA na organizmy zwierząt i człowieka [Flint i in. 2012; Jeong i in. 2017; Lee i in. 2018; Shafei i in. 2018]. Liczne wyniki badań epidemiologicznych wskazały na zależność między podwyższonym poziomem BPA w organizmie, a zwiększonym ryzykiem rozwoju otyłości, cukrzycy, chorób serca, alergii czy astmy [Shankar i Teppala 2011; Shankar i in. 2012; Wang i in. 2012; Kim i in. 2014; Gao i Wang 2014; Hong i in. 2017]. Ze względu na estrogenne właściwości BPA, uważa się, że związek ten obniża płodność oraz powoduje działanie teratogenne [Vandenberg 2011; Laing i in. 2016].

Doniesienia na temat szkodliwych efektów wywieranych przez BPA stały się bodźcem do wprowadzenia regulacji prawnych dotyczących ograniczenia bądź zakazania stosowania tej substancji w produktach codziennego użytku. Przepisy regulujące zakaz stosowania BPA w produktach dla niemowląt oraz w opakowaniach na żywność i butelkach na wodę pitną wdrożyło wiele państw m.in. Kanada, USA oraz kraje Unii Europejskiej [Liao i Kannan 2013]. Wprowadzenie ograniczeń w stosowaniu BPA przyczyniło się do wzrostu produkcji innych bisfenoli stosowanych jako zamienniki dla tej substancji, w tym bisfenolu S (BPS; 4,4'-dihydroksy-difenylosulfon), bisfenolu F (BPF; 1,1-bis-(4-hydroksyfenylometan)) i bisfenolu AF (BPAF; 2,2-bis-(4-hydroksyfenyl) heksafluoro-propan)).

Liczba badań opisujących oddziaływanie substytutów (analogów) BPA na układy biologiczne jest dalece niewystarczająca, dlatego nie można stwierdzić, czy związki te są mniej szkodliwe niż BPA. BPS, BPF i BPAF są analogami strukturalnymi BPA, a zatem mogą wywierać podobne efekty biologiczne [Song i in. 2014]. Grignard i in. [2012] oraz Cabaton i in. [2009] wykazali, że BPS i BPF wykazują porównywalny potencjał estrogenny do BPA, podczas gdy Ruan i in. [2014] stwierdzili, że BPAF charakteryzuje się silniejszym potencjałem estrogenowym niż BPA. Wykazano również, że zamienniki BPA mogą charakteryzować się działaniem rakotwórczym [Kim i in. 2017]. W badaniach stwierdzono ponadto, że BPAF wywiera silniejszy wpływ nekrotyczny, apoptotyczny, oksydacyjny i genotoksyczny niż BPA względem jednojądrzastych komórek krwi obwodowej człowieka [Michałowicz i in. 2015; Mokra i in. 2015; Mokra i in. 2017]. Opisywane analogi BPA oznaczono w środowisku naturalnym, żywności, wodzie pitnej i kurzu pomieszczeń mieszkalnych [Viñas i in. 2010; Liao i in. 2012]. BPS, BPF i BPAF oznaczono także w ludzkiej krwi i moczu zarówno osób narażonych środowiskowo, jak i zawodowo na te substancje [Song i in. 2014; Yang i in. 2014; Rocha i in. 2016].

CEL PRACY

Niewiele prac badawczych poświęcono analizie wpływu BPA i jego analogów na komórki krwi. Dotychczas, przeprowadzono tylko jedno badanie dotyczące oddziaływania BPA na erytrocyty człowieka jako komórki w dużym stopniu narażone na ksenobiotyki [Suthar i in. 2014]. Ponadto, nie wykonano jakichkolwiek analiz oceniających wpływ analogów BPA na krwinki czerwone. Zważywszy na przewlekłe, wciąż narastające ogólnopopulacyjne narażenie ludzi na bisfenole, podjęcie tego typu badań jest bardzo istotne.

Celem badawczym niniejszej rozprawy doktorskiej było określenie wpływu BPA oraz jego wybranych analogów, tj.: BPS, BPF i BPAF na erytrocyty człowieka. Analizowano wpływ tych substancji chemicznych na proces hemolizy, parametry morfologiczne oraz apoptotyczne badanych komórek. Określono również oddziaływanie BPA i jego analogów na parametry stresu oksydacyjnego, aktywność systemu antyoksydacyjnego oraz błonę komórkową krwinek czerwonych.

OMÓWIENIE PRAC WCHODZĄCYCH W ZAKRES ROZPRAWY DOKTORSKIEJ

Na niniejszą rozprawę doktorską składają się cztery publikacje stanowiące spójny tematycznie cykl artykułów. Każdy artykuł został opublikowany w indeksowanym czasopiśmie naukowym o międzynarodowym zasięgu, znajdującym się w bazie *Journal Citation Reports*. Seria prac jest odzwierciedleniem założonego pierwotnie planu badań, który finalnie został w pełni zrealizowany.

Cykl artykułów otwiera publikacja pt. „*Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes"*(**I**). W pracy porównano wpływ BPA i jego wybranych analogów (BPS, BPF, BPAF) na proces hemolizy, parametry morfologiczne oraz utlenianie hemoglobiny (powstawanie methemoglobiny) w erytrocytach człowieka.

Zmiany hemolityczne (żywotność) oznaczono spektrofotometrycznie poprzez pomiar ilości hemoglobiny uwolnionej z erytrocytów. Metodę spektrofotometryczną użyto również do określenia zmian w poziomie methemoglobiny. Do oceny zmian w wielkości komórek wykorzystano technikę cytometrii przepływowej, natomiast analizę kształtu krwinek czerwonych przeprowadzono z użyciem mikroskopii z kontrastem fazowym.

Zaobserwowano, że badane związki charakteryzują się różnym potencjałem hemolitycznym i oksydacyjnym. BPA wykazywał najsilniejszy potencjał oksydacyjny (utlenianie hemoglobiny), podczas gdy BPAF charakteryzował się najsilniejszą cytotoksycznością oraz indukował najbardziej znaczące zmiany morfologiczne w ludzkich erytrocytach. Znacznie niższą toksyczność w porównaniu do BPA wykazywał BPS, stanowiący główny substytut bisfenolu A w produkcji polimerów i papieru termicznego. Analiza przeprowadzona techniką mikroskopii wykazała, że BPA i BPAF indukowały powstawanie echinocytów, natomiast w wyniku działania BPF obserwowano tworzenie się stomatocytów. W oparciu o uzyskane wyniki można stwierdzić, że BPA i jego analogi spowodowały zróżnicowane zmiany w stopniu hemolizy, morfologii i utlenianiu hemoglobiny w erytrocytach człowieka, co wynikało najprawdopodobniej z różnej hydrofobowości (lipofilności) i reaktywności (obecność różnych podstawników) tych substancji chemicznych. Należy podkreślić, że zmiany hemolityczne i oksydacyjne odnotowano pod wpływem bisfenoli w stężeniach, które mogą oddziaływać na organizm człowieka wskutek narażenia zawodowego lub w wyniku zatrucia podostrego tymi substancjami.

W drugiej pracy pt. „*Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study)*” (**II**) analizowano zmiany apoptotyczne w erytrocytach człowieka poddanych działaniu BPA, BPS, BPF i BPAF.

Wykorzystując technikę cytometrii przepływowej dokonano analizy zmian w poziomie jonów wapnia w cytozolu komórek (użycie sondy fluorescencyjnej Fluo-3-AM), eksternalizacji fosfatydyloseryny (użycie aneksyny V sprzężonej z fluoresceiną) oraz zmian w aktywności kaspazy-3 (wykorzystanie substratu DEVD-FMK skoniugowanego z fluoresceiną) i aktywności kalpain (zastosowanie substratu t-butoksykarbonylo-Leu-Met sprzężonego z 7-amino-4-chloro-metylo-kumaryną).

Przeprowadzone badania wykazały, że BPA i jego analogi charakteryzuje odmienny potencjał apoptotyczny względem erytrocytów człowieka. Analizowane związki podwyższały poziom jonów wapnia w cytozolu, przy czym najsilniejszy efekt spowodował BPAF. Stwierdzono również, że wszystkie badane bisfenole, a w szczególności BPAF i BPF, zwiększały translokację fosfatydyloseryny w badanych komórkach. Wykazano ponadto, że badane związki wzmagały aktywność kaspazy-3 i kalpain, przy czym najsilniejszy efekt odnotowano pod wpływem BPAF, natomiast BPS i BPA wykazywały porównywalny potencjał eryptotyczny. Na podstawie uzyskanych wyników można wnioskować, że BPA, BPS, BPF i BPAF indukują proces eryptozy w krwinkach czerwonych człowieka poprzez podwyższenie poziomu jonów wapnia w cytozolu, eksternalizację fosfatydyloseryny oraz aktywację kaspazy-3 i kalpain. Zmiany eryptotyczne w ludzkich erytrocytach zachodziły pod wpływem bisfenoli w stężeniach, które mogą wpływać na organizm człowieka podczas ekspozycji zawodowej lub podostrego zatrucia tymi związkami.

Artykuł zatytułowany „*Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study)*” (**III**) dotyczył oceny oddziaływania BPA i jego analogów (BPS, BPF, BPAF) na parametry stresu oksydacyjnego oraz aktywność systemu antyoksydacyjnego w erytrocytach człowieka.

Analiza cytometryczna umożliwiła ocenę zmian w poziomie reaktywnych form tlenu (RFT) (użycie znacznika fluorescencyjnego dioctanu 5’-karboksy-2’,7’-dichlorodihydrofluoresceiny) oraz wysoce reaktywnych form tlenu, głównie rodnika hydroksylowego (użycie sondy fluorescencyjnej 3'-(p-hydroksyfenylo)fluoresceiny). Peroksydację lipidów analizowano spektrokolorymetrycznie określając zawartość substancji reagujących z kwasem tiobarbiturowym (TBARS) [Stocks i Dormandy 1971].

Analiza spektrofotometryczna pozwoliła ocenić zmiany w poziomie glutationu zredukowanego (GSH) [Ellman 1959] oraz zmiany w aktywności enzymów antyoksydacyjnych, tj.: dysmutazy ponadtlenkowej (SOD) [Misra i Fridovich 1972], peroksydazy glutationowej (GSH-Px) [Rice-Evans i in. 1991] i katalazy (CAT) [Aebi 1984].

Wykazano, że BPA, BPS, BPF i BPAF odznaczają się zróżnicowanym potencjałem prooksydacyjnym – co zaobserwowano już w pierwszej publikacji, analizując stopień utlenienia hemoglobiny (**I**). Badane związki podwyższały poziom RFT (w tym rodnika hydroksylowego), obniżały poziom GSH, indukowały proces peroksydacji lipidów oraz zmieniały (głównie obniżały) aktywność enzymów antyoksydacyjnych (SOD, GSH-Px, CAT). Stwierdzono, że BPA, a szczególnie BPAF wykazywały najsilniejszy potencjał oksydacyjny, natomiast BPS nie zmieniał większości badanych parametrów. Podobnie, jak i w uprzednio opisanych pracach, analizowane parametry ulegały zmianom pod wpływem bisfenoli w stężeniach, które mogą oddziaływać na organizm człowieka wskutek narażenia zawodowego (BPA, BPAF) lub/oraz zatrucia podostrego. W oparciu o otrzymane wyniki, można stwierdzić, że BPA, BPF i BPAF indukują stres i uszkodzenia oksydacyjne oraz wpływają na system antyoksydacyjny w erytrocytach człowieka.

Badania zaprezentowane w ostatniej publikacji pt. „*The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities*” (**IV**) miały na celu określenie wpływu BPA i jego analogów (BPS, BPF, BPAF) na błonę komórkową erytrocytów człowieka.

Płynność błony analizowano z użyciem spektroskopii elektronowego rezonansu paramagnetycznego (EPR) z zastosowaniem dwóch wyznakowanych kwasów tłuszczowych, tj.: kwasu 5-doksylostearynowego (5-DSA) i kwasu 16-doksylo-stearynowego (16-DSA). Tę samą technikę wykorzystano do oceny zmian w parametrze W/S – zmian konformacyjnych białek błonowych (użycie znacznika 4-maleimido-2,2,6,6-tetrametylopiperydyno-1-oksylu) oraz pomiaru mikrolepkości wnętrza erytrocytu (wykorzystanie znacznika 4-amino-2,2,6,6-tetrametylopiperydyno-1-oksylu). Do pomiaru oporności osmotycznej krwinek czerwonych zastosowano technikę spektrokolorymetryczną (pomiar stopnia hemolizy). Ocenę oksydacyjnych uszkodzeń białek oparto na pomiarze fluorescencji tryptofanu z zastosowaniem analizy fluorymetrycznej. Poziom wewnątrzkomórkowego ATP określono na podstawie pomiaru

bioluminescencji w reakcji utleniania lucyferyny przez lucyferazę. Analizę spektrofotometryczną wykorzystano do oceny poziomu grup tiolowych (-SH), aktywności acetylocholinoesterazy (AChE) [Ellman 1961] oraz aktywności ATPazy Na^+/K^+ [Bartosz 1994].

Otrzymane wyniki wykazały, że BPA, BPS, BPF i BPAF w różnym stopniu indukują zmiany w błony komórkowej erytrocytów człowieka. Wykazano, że analizowane związki (z wyjątkiem BPS) zmieniały płynność błony komórkowej w głębszym rejonie jej monowarstwy, zwiększały mikrolepkość wnętrza erytrocytu oraz poziom grup tiolowych, a także obniżały poziom ATP w komórce. Wszystkie badane bisfenole zwiększały oporność osmotyczną erytrocytów oraz zmieniały stan konformacyjny białek błonowych. Ponadto, BPA i jego analogi spowodowały oksydacyjne uszkodzenia białek błonowych, obniżały aktywność ATPazy Na^+/K^+ oraz zmieniały aktywność acetylocholinoesterazy. Wykazano, że BPAF spowodował najsilniejsze zmiany w większości badanych parametrów, podczas gdy BPS w niewielkim stopniu (lub wcale) wywierał wpływ na błonę erytrocytu. Obserwowane zmiany w błonie komórkowej erytrocytów zachodziły pod wpływem bisfenoli w stężeniach, które mogą oddziaływać na organizm człowieka w warunkach narażenia zawodowego.

Na podstawie uzyskanych wyników (z uwzględnieniem zastosowanych stężeń związków, analizowanych parametrów oraz badanego typu komórek) można wnioskować, że BPS wykazuje słabszą toksyczność w porównaniu do BPA, co może wskazywać na zasadność stosowania tej substancji jako substytutu bisfenolu A w produkcji tworzyw sztucznych mających kontakt z żywnością i wodą pitną. BPAF jako związek wykazujący najsilniejszy potencjał toksyczny względem badanych komórek, nie powinien być wykorzystywany jako zamiennik BPA w produkcji przemysłowej.

WNIOSKI

- Bisfenole, a w szczególności BPAF wzmagały hemolizę erytrocytów człowieka, co wiązało się m.in. z obniżeniem poziomu wewnątrzkomórkowego ATP; badane związki (z wyjątkiem BPS) spowodowały znaczące zmiany morfologiczne, wywołując echinocytozę (BPA, BPAF) i stomatocytozę (BPF)

- BPA i jego analogi wykazywały zróżnicowany potencjał eryptotyczny, zwiększając poziom jonów wapnia w cytozolu, wzmagając eksternalizację fosfatydyloseryny oraz aktywując kaspazę-3 i kalpainy

- BPA, BPF i BPAF charakteryzowały się znaczącym potencjałem oksydacyjnym; związki te generowały RFT, utleniały hemoglobinę, indukowały uszkodzenia oksydacyjne białek i lipidów oraz zmieniały funkcjonowanie systemu antyoksydacyjnego

- BPA i jego analogi wpływały na wzrost płynności hydrofobowych rejonów dwuwarstwy lipidowej błony (poza BPS), wzmagały zmiany konformacyjne białek błonowych oraz oporność osmotyczną erytrocytu, a ponadto zmieniały aktywność ATP-azy Na^+/K^+ i AChE

- Uzyskane wyniki (w zakresie analizowanych parametrów oraz badanego typu komórek) wskazują na zasadność zastępowania BPA przez BPS w produkcji artykułów przemyslowych, natomiast poddają w wątpliwość stosowanie w tym celu BPAF

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3. STRESZCZENIE W JĘZYKU POLSKIM

Bisfenol A (BPA) jest związkiem chemicznym powszechnie stosowanym w produkcji poliwęglanów, żywic epoksydowych i papieru termicznego. Badania toksykologiczne wykazały niekorzystny wpływ BPA na organizm człowieka. Dowiedziono, że związek ten charakteryzuje się działaniem toksycznym, estrogennym oraz potencjalnie kancerogennym. Uważa się także, że wpływ BPA na organizm ludzki może prowadzić do rozwoju otyłości, cukrzycy, chorób serca, alergii czy astmy. Dane wskazujące na toksyczność BPA oraz jego obecność w organizmie człowieka, skutkują sukcesywnym zastępowaniem tego związku w produkcji przemysłowej przez liczne substytuty (analogi), w tym bisfenol S (BPS), bisfenol F (BPF) oraz bisfenol AF (BPAF).

W wielu badaniach epidemiologicznych wykazano obecność BPA i jego analogów w organizmie człowieka (tkanki stałe, krew, mocz). Mając na uwadze przewlekłe narażenie populacji ogólnej na bisfenole, kontakt tych związków z erytrocytami w układzie krwionośnym oraz brak prac opisujących wpływ BPA i jego analogów na ten typ komórek, istnieje konieczność poznania mechanizmów oddziaływania tych substancji chemicznych na krwinki czerwone.

Celem niniejszej pracy było określenie wpływu BPA i jego wybranych analogów, tj.: BPS, BPF i BPAF na erytrocyty człowieka. Analizowano wpływ bisfenoli na parametry hemolityczne, morfologiczne i apoptotyczne. Określono także uszkodzenia oksydacyjne, zmiany w aktywności systemu antyoksydacyjnego oraz zmiany w błonie komórkowej krwinek czerwonych poddanych działaniu tych związków.

W badaniach wykorzystano różne techniki analityczne, w tym spektrofotometrię, spektrofluorymetrię, cytofluorymetrię, mikroskopię kontrastu fazowego i spektroskopię elektronowego rezonansu paramagnetycznego (EPR).

Wykazano, że badane związki spowodowały hemolizę oraz utleniały hemoglobinę w erytrocytach człowieka. BPAF indukował najsilniejsze zmiany hemolityczne, podczas gdy BPA charakteryzował się najsilniejszym potencjałem oksydacyjnym. Analiza przeprowadzona metodą cytometrii przepływowej wykazała, że wszystkie badane związki spowodowały istotne zmiany w morfologii (wielkości) erytrocytów człowieka. Przy użyciu mikroskopu kontrastowo-fazowego zaobserwowano, że bisfenole zmieniały kształt erytrocytów, powodując powstanie echinocytów (BPA i BPAF) oraz stomatocytów (BPF).

Stwierdzono także, iż BPS nie spowodował znaczących zmian w wielkości i kształcie komórek.

Wyniki badań wykazały także, że analizowane substancje chemiczne charakteryzuje zróżnicowany potencjał apoptotyczny względem ludzkich erytrocytów. BPA i jego analogi (szczególnie BPAF) podwyższały poziom jonów wapnia w cytozolu, spowodowały translokację fosfatydyloseryny oraz wzmagały aktywność kaspazy-3 i kalpain w krwinkach czerwonych. Największe zmiany eryptotyczne odnotowano pod wpływem BPAF, natomiast BPA i BPS wykazywały porównywalny potencjał apoptotyczny.

Stwierdzono ponadto, że badane związki wzmagały tworzenie reaktywnych form tlenu (RFT) (w tym rodnika hydroksylowego), obniżały poziom zredukowanego glutationu (GSH), zwiększały peroksydację lipidów oraz zmieniały (głównie obniżały) aktywność enzymów antyoksydacyjnych tj.: dysmutazy ponadtlenkowej (SOD), katalazy (CAT) i peroksydazy glutationowej (GSH-Px). Stwierdzono, że najsilniejsze zmiany zachodziły pod wpływem BPAF i BPA, natomiast BPS nie zmieniał większości badanych parametrów.

W oparciu o wyniki eksperymentów dotyczących wpływu BPA, BPS, BPF i BPAF na błonę komórkową erytrocytów stwierdzono, że analizowane związki zmieniały płynność błony (w części hydrofobowej dwuwarstwy lipidowej), zwiększały mikrolepkość wnętrza erytrocytu oraz wzmagały oporność osmotyczną krwinek czerwonych. Ponadto, BPA i jego analogi zmieniały konformację białek błonowych, podwyższały poziom grup tiolowych, indukowały oksydacyjne uszkodzenia białek błonowych, obniżały poziom wewnątrzkomórkowego ATP, zmniejszały aktywność ATPazy Na^+/K^+, a także zmieniały aktywność acetylocholinoesterazy (AChE). Najsilniejsze zmiany w analizowanych parametrach obserwowano pod wpływem BPAF, najsłabsze zaś w wyniku działania BPS.

Wyniki badań wskazują, że bisfenole spowodowały zmiany w erytrocytach człowieka w stężeniach, które mogą oddziaływać na organizm ludzki w warunkach narażenia zawodowego lub na skutek zatrucia podostrego tymi substancjami. Przeprowadzone badania wskazują także (w odniesieniu do badanego typu komórek i przeprowadzonych analiz) na zasadność wykorzystywania BPS jako substytutu BPA w produkcji tworzyw sztucznych i innych materiałów użytkowych, natomiast podważają zasadność stosowania w tym celu BPAF.

4. STRESZCZENIE W JĘZYKU ANGIELSKIM

Bisphenol A (BPA) is a chemical substance widely used in the manufacturing of polycarbonates, epoxy resins and thermal paper. Toxicological studies have shown that BPA can cause adverse health effects in human organism. It has been proven that BPA exhibits toxic, estrogenic and potentially carcinogenic potential. Furthermore, BPA is suspected to increase risk of obesity, diabetes, heart diseases, allergy and asthma in humans. Due to significant toxicity of BPA and its presence in the human body, this chemical is successively substituted in the industry by its numerous analogs, including bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF). Many epidemiological surveys have also determined BPA analogs in tissues and fluids of human body including blood and urine, which shows common exposure of the general population to their action.

Taking into consideration chronic exposure of humans to BPA and its analogs, significant contact of erythrocytes with bisphenols in the circulation and lack of studies evaluating the effect of BPA and its analogs on human erythrocytes, it is necessary to explain the mechanisms of action of these substances on human red blood cells, which are crucial for proper function of human organism.

The purpose of this study was to evaluate the effect of BPA and its selected analogs, i.e. BPS, BPF and BPAF on human erythrocytes. The impact of these substances on hemolytic, morphological and eryptotic parameters has been assessed. Moreover, the effect of BPA and its analogs on oxidative stress parameters, function of antioxidant system and erythrocyte membrane has been evaluated.

Various analytical techniques including spectrophotometry, spectrofluorimetry, cytometry, phase-contrast microscopy and electron paramagnetic resonance have been employed in this study.

The compounds examined caused hemolysis and hemoglobin oxidation in human erythrocytes. BPAF induced the strongest hemolytic effect, while BPA exhibited the highest oxidative potential. Flow cytometry analysis has shown that all bisphenols studied (excluding BPS) significantly altered erythrocytes morphology (cell size). The use of phase-contrast microscopy has revealed that BPA and BPAF induced echinocytes formation, BPF caused stomatocytes creation, while BPS did not exert significant changes in the shape of red blood cells.

The results achieved using flow cytometry have also shown that the substances examined exhibited different eryptotic potential. BPA and its analogs (particularly BPAF) increased cytosolic calcium ions level and phosphatidylserine translocation in human erythrocytes. Furthermore, it has been found that bisphenols studied caused significant increase in caspase-3 and calpain activities. The strongest eryptotic effects were noted for BPAF, while BPS exhibited comparable apoptotic potential to BPA.

It has also been shown that bisphenols examined enhanced reactive oxygen species (ROS) (including hydroxyl radical) formation, depleted the level of reduced glutathione (GSH), increased lipid peroxidation and changed (mainly decreased) the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). It has been noted that the strongest alterations were induced by BPAF and BPA, while BPS did not alter most parameters studied.

Moreover, the study has demonstrated that the compounds studied changed membrane fluidity at its hydrophobic region, increased internal viscosity and osmotic fragility of the erythrocytes and changed conformational state of membrane proteins. BPA and its analogs have also increased thiol groups level, induced oxidative damage to membrane proteins, decreased intracellular ATP level, depleted Na^+/K^+ ATPase activity and changed acetylcholinesterase (AChE) activity in red blood cells.

Observed changes in human erythrocytes were provoked by BPA and its analogs at the concentrations, which may influence the human organism during occupational exposure or subacute poisoning with these compounds. The obtained results (within the cell type studied and the parameters examined) have shown that BPS may be applied as a substitute of BPA in the production of polymers and other materials, while BPAF should not be used for these purposes.

5. DOROBEK NAUKOWY

PUBLIKACJE

Publikacje wchodzące w skład rozprawy doktorskiej

Maćczak A., Bukowska B., Michałowicz J. *Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes.* Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 2015, 176-177: 62-70.

Praca oryginalna

Punkty MNiSW: **30**

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Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study).* Journal of Hazardous Materials 2016, 307: 328-335.

Praca oryginalna

Punkty MNiSW: **45**

Impact Factor: **6,065**, pięcioletni Impact Factor: **6,393**

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study).* Toxicology in Vitro 2017, 41: 143-149.

Praca oryginalna

Punkty MNiSW: **30**

Impact Factor: **2,866**, pięcioletni Impact Factor: **3,103**

Maćczak A., Duchnowicz P., Sicińska P., Koter-Michalak M., Bukowska B., Michałowicz J. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities.* Food and Chemical Toxicology 2017, 110: 351-359.

Praca oryginalna

Punkty MNiSW: **40**

Impact Factor: **3,778**, pięcioletni Impact Factor: **3,628**

Pozostałe publikacje

Sicińska P., Pytel E., **Maćczak A.**, Koter-Michalak M. 2015. *Zastosowanie różnych suplementów diety w zespole metabolicznym*. Postępy Higieny i Medycyny Doświadczalnej, 69: 25-33.

Praca przeglądowa

Punkty MNiSW: **15**

Impact Factor: **0,769**, pięcioletni Impact Factor: **0,795**

Michałowicz J., Wróblewski W., Mokra K., **Maćczak A**., Kwiatkowska M. 2015. *Comparative study of the effect of chloro-, dichloro- bromo-, and dibromoacetic acid on necrotic, apoptotic and morphological changes in human peripheral blood mononuclear cells (in vitro study).* Toxicology in Vitro, 29: 1416-1424.

Praca oryginalna

Punkty MNiSW: **30**

Impact Factor: **3,338**, pięcioletni Impact Factor: **3,103**

Maćczak A., Bąk A., Michałowicz J. 2014. *Ocena oddziaływania wybranych bisfenoli na leukocyty człowieka*. Rozdział w monografii pt. „Diagnozowanie Stanu Środowiska, Metody Badawcze-Prognozy". Red. Jerzy K. Garbacz. BTN, Bydgoszcz, 8: 155-164.

Praca przeglądowa

Punkty MNiSW: **4**

Bąk A., **Maćczak A**., Michałowicz J. 2014. *Analiza oddziaływania chlorofenoli na zmiany nekrotyczne i morfologiczne granulocytów człowieka*. Rozdział w monografii pt. „Diagnozowanie Stanu Środowiska, Metody Badawcze-Prognozy". Red. Jerzy K. Garbacz. BTN, Bydgoszcz, 8: 7-15.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A., Mokra K. *Żywność jako główne źródło narażenia na bisfenole*. 2015. Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Żywność i Bisfenole". Tom I, str. 50-54, wyd. Młodzi Naukowcy, ISBN 978-83-65362-05-6.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A., Mokra K. *Bisfenol S – bezpieczny substytut dla bisfenolu A*? 2015. Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Żywność i Bisfenole". Tom I, str. 55-60, wyd. Młodzi Naukowcy, ISBN 978-83-65362-05-6.

Praca przeglądowa

Punkty MNiSW: **4**

Mokra K., **Maćczak A**. *Wpływ bisfenolu A i jego pochodnych na układ hormonalny człowieka*. 2015. Rozdział w monografii pt. "Badania i Rozwój Młodych Naukowców w Polsce. Żywność i Bisfenole". Tom I, str. 61-66, wyd. Młodzi Naukowcy, ISBN 978-83-65362-05-6.

Praca przeglądowa

Punkty MNiSW: **4**

Mokra K., **Maćczak A**. *Biotransformacja bisfenolu A i bisfenolu F w organizmie człowieka.* 2015. Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Żywność i Bisfenole". Tom I, str. 67-73, wyd. Młodzi Naukowcy, ISBN 978-83-65362-05-6.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A., Cyrkler M. 2016. *Endocrine Disrupting Compounds (EDCs).* Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Nauki medyczne i nauki o zdrowiu". Część III, str. 57-62, wyd. Młodzi Naukowcy, ISBN 978-83-65362-26-1.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A., Cyrkler M. 2016. *The presence of BPA and its selected analogues in human organism*. Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Nauki medyczne i nauki o zdrowiu". Część III, str. 63-67, wyd. Młodzi Naukowcy, ISBN 978-83-65362-26-1.

Praca przeglądowa

Punkty MNiSW: **4**

Cyrkler M., **Maćczak A**. 2016. *Characteristic of flame retardants.* Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Weterynaria i botanika". str. 73-77, wyd. Młodzi Naukowcy, ISBN 78-83-65362-13-1.

Praca przeglądowa

Punkty MNiSW: **4**

Cyrkler M., **Maćczak A**. 2016. *The erythrocytes as a basic experimental model.* Rozdział w monografii pt. „Badania i Rozwój Młodych Naukowców w Polsce. Weterynaria i botanika". str. 78-82, wyd. Młodzi Naukowcy, ISBN 78-83-65362-13-1.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A. 2017. *The carcinogenic effects of bisphenol A.* Rozdział w monografii pt. "Badania i Rozwój Młodych Naukowców w Polsce. Nauki przyrodnicze". Część IV, str. 62-66, wyd. Młodzi Naukowcy, ISBN 978-83-65677-19-8.

Praca przeglądowa

Punkty MNiSW: **4**

Maćczak A. 2017. *Genotoxic and epigenetic properties of bisphenols.* Rozdział w monografii pt. ”Badania i Rozwój Młodych Naukowców w Polsce. Nauki przyrodnicze”. Część IV, str. 67-71, wyd. Młodzi Naukowcy, ISBN 978-83-65677-19-8.

Praca przeglądowa

Punkty MNiSW: **4**

Sumaryczna wartość **IF** dorobku publikacyjnego łącznie z publikacjami wchodzącymi w skład rozprawy doktorskiej (wg roku opublikowania) wynosi **19,362 (238 punktów MNiSW)**.

KOMUNIKATY KONFERENCYJNE

Maćczak A., Bukowska B., Michałowicz J. *Hemoliza i utlenianie hemoglobiny w erytrocytach człowieka inkubowanych z bisfenolem A i S.* Człowiek, żywność, środowisko – problemy współczesnej toksykologii, Olsztyn 16-19 wrzesień 2014.

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Wpływ bisfenolu F i bisfenolu AF na hemolizę, utlenianie hemoglobiny i morfologię erytrocytów człowieka.* IV Konferencja Biologii Molekularnej, Łódź 26-28 marzec 2015.

Cyrkler M., **Maćczak A**., Bukowska B., Michałowicz J. *Ocena oddziaływania wybranych bisfenoli na poziom glutationu i reaktywnych form tlenu w erytrocytach człowieka*. IV Konferencja Biologii Molekularnej, Łódź 26-28 marzec 2015.

Maćczak A., Cyrkler M., Mokra K., Bukowska B., Michałowicz J. *Ocena oddziaływania BPA i jego analogów na hemolizę, utlenianie hemoglobiny oraz parametry morfologiczne erytrocytów człowieka.* I Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 20-22 kwiecień 2015.

Mokra K., Bąk A., **Maćczak A**., Kocia M., Michałowicz J. *Wpływ bisfenolu A i jego analogów na zmiany wybranych parametrów oksydacyjnych w jednojądrzastych komórkach krwi człowieka*. I Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 20-22 kwiecień 2015.

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Bisfenol A a rozwój otyłości.* III Studencka Konferencja Biologii Medycznej Biofuzje, Warszawa 22-24 maj 2015.

Maćczak A., Mokra K., Cyrkler M., Bukowska B., Michałowicz J. *Zmiany apoptotyczne w erytrocytach człowieka pod wpływem bisfenolu AF.* Warsztaty Naukowe Nowe Trendy w Toksykologii: Trwałe zanieczyszczenia organiczne – zagrożenia dla zdrowia i środowiska, Łódź 25-27 maj 2015.

Mokra K., **Maćczak A**., Cyrkler M., Michałowicz J. *Indukcja apoptozy pod wpływem bisfenolu AF w jednojądrzastych komórkach krwi człowieka*. Warsztaty Naukowe Nowe Trendy w Toksykologii: Trwałe zanieczyszczenia organiczne – zagrożenia dla zdrowia i środowiska, Łódź 25-27 maj 2015.

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Ocena aktywności enzymów antyoksydacyjnych w erytrocytach człowieka poddanych działaniu bisfenolu A i bisfenolu S.* Międzyuczelniane Sympozjum Biotechnologiczne Symbioza, Warszawa 29-31 maj 2015.

Maćczak A., Mokra K., Cyrkler M., Bukowska B., Michałowicz J. *Indukcja eryptozy pod wpływem bisfenolu F w erytrocytach człowieka.* Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce – Badania i Rozwój, Poznań 8 czerwiec 2015.

Mokra K., **Maćczak A**., Kocia M., Michałowicz J. *Indukcja apoptozy i nekrozy pod wpływem bisfenolu F w jednojądrzastych komórkach krwi człowieka*. Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce – Badania i Rozwój, Poznań 8 czerwiec 2015.

Bukowska B., **Maćczak A**., Cyrkler M., Mokra K., Michałowicz J. *Zmiany apoptotyczne w erytrocytach człowieka pod wpływem bisfenolu A i bisfenolu S.* 58 Zjazd Naukowy Polskiego Towarzystwa Chemicznego w Gdańsku, 21-25 wrzesień 2015.

Michałowicz J., Mokra K., Kocia M., **Maćczak A**., Bukowska B. *Zmiany apoptotyczne pod wpływem bisfenolu S w jednojądrzastych komórkach krwi człowieka.* 58 Zjazd Naukowy Polskiego Towarzystwa Chemicznego w Gdańsku, 21-25 wrzesień 2015.

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *The effect of bisphenol A and bisphenol S on antioxidant enzymes activities in human erythrocytes*. VII International Conference of Biotechnology Students & XVII National Academic Seminar of Biotechnology Students, Poznań 20-22 listopad 2015.

Maćczak A., Cyrkler M., Bukowska B., Michałowicz J. *Aktywacja kaspazy-3 i kalpain w erytrocytach człowieka inkubowanych z bisfenolem A i jego wybranymi analogami*. II Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce – Badania i Rozwój, Wrocław 25 listopad 2015.

Maćczak A., Bukowska B., Michałowicz J. *The effect of bisphenol A and its selected analogues on antioxidant enzymes activity in human erythrocytes*. 17th International Conference on Life Science, Biomedical and Biological Engineering, Rzym 3-4 grudzień 2015.

Maćczak A., Kolmaga P., Bukowska B., Michałowicz J. *Wpływ wybranych bisfenoli na poziom glutationu i reaktywnych form tlenu w erytrocytach człowieka*. II Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 12-14 maj 2016.

Cyrkler M., **Maćczak A**., Michałowicz J., Bukowska B. *Ocena oddziaływania bisfenolu S oraz tetrabromobisfenolu S na hemolizę oraz utlenianie hemoglobiny erytrocytów człowieka*. III Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce – Badania i Rozwój, Gdańsk 18 kwiecień 2016.

Maćczak A., Koter-Michalak M., Duchnowicz P., Bukowska B., Michałowicz J. *The effect of bisphenol A and its selected analogs on cell membrane fluidity of human erythrocytes.* XVI Zjazd Polskiego Towarzystwa Biofizycznego, Ryn 28 czerwiec-1 lipiec 2016.

Maćczak A., Bukowska B., Michałowicz J. *The effect of bisphenol AF on lipid peroxidation and antioxidant enzymes activity in human erythrocytes*. 52nd European Congress of the European Societies of Toxicology, Sewilla 4-7 wrzesień 2016.

Maćczak A., Bukowska B., Michałowicz J. *The presence of bisphenol A in the environment.* XLI Międzynarodowe Seminarium Naukowo-Techniczne „Chemistry for Agriculture", Karpacz 27-30 listopad 2016.

Maćczak A., Bukowska B., Michałowicz J. *Bisfenol S jako alternatywa dla bisfenolu A.* V Ogólnokrajowa Konferencja Młodzi Naukowcy w Polsce – Badania i Rozwój, Poznań 8 maj 2017 (prezentacja ustna).

Maćczak A., Jarosiewicz M., Włuka A., Bukowska B., Michałowicz J. *Zmiany aktywności acetylocholinoesterazy w erytrocytach człowieka pod wpływem bisfenolu A i jego wybranych analogów*. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 11-12 maj 2017.

Jarosiewicz M., **Maćczak A**., Włuka A., Bukowska B. *Ocena oddziaływania tetrabromobisfenolu S na hemolizę, utlenianie hemoglobiny oraz aktywność acetylocholinoesterazy w erytrocytach człowieka.* III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 11-12 maj 2017.

Włuka A., Jarosiewicz M., **Maćczak A**., Michałowicz J. *Ocena wpływu fenolu oraz pentachlorofenolu na apoptozę erytrocytów człowieka.* III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 11-12 maj 2017.

Maćczak A., Duchnowicz P., Koter-Michalak M., Sicińska P., Bukowska B., Włuka A., Michałowicz J. *The effect of bisphenol AF on human erythrocyte membrane*. III International Conference of Cell Biology, Kraków 26-27 maj 2017.

Włuka A., Jarosiewicz M., **Maćczak A**., Michałowicz J. *Ocena wpływu 2,4-dichlorofenolu oraz 2,4,6-trichlorofenolu na apoptozę erytrocytów człowieka*. VI Międzyuczelniane Sympozjum Biotechnologiczne Symbioza, Warszawa 26-28 maj 2017.

Maćczak A., Duchnowicz P., Sicińska P., Koter-Michalak M., Bukowska B., Michałowicz J. *The effect of bisphenol A and its analogs on human erythrocyte membrane*. XLII Międzynarodowe Seminarium Naukowo-Techniczne „Chemistry for Agriculture", Karpacz 26-29 listopad 2017.

Maćczak A., Włuka A., Jarosiewicz M., Michałowicz J. *The effect of phenol and pentachlorophenol on selected apoptotic parameters of human erythrocytes*. XLII Międzynarodowe Seminarium Naukowo-Techniczne „Chemistry for Agriculture", Karpacz 26-29 listopad 2017.

6. OŚWIADCZENIA WSPÓŁAUTORÓW PRAC

Łódź, dn. 5.03.2018 r.

Mgr Aneta Wolska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Bożena Bukowska., Jaromir Michałowicz. 2015. *Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes.* Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 176-177: 62-70.

Oświadczam, że mój wkład w powstawanie publikacji polegał na pomocy w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Wykonałam wszystkie analizy dotyczące zmian hemolitycznych i morfologicznych oraz utleniania hemoglobiny w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Ponadto, dokonałam interpretacji większości uzyskanych wyników i napisałam publikację. Mój udział oceniam na **60%**.

.....Aneta...Wolska...(Maćczak).....

Łódź, dn. 5.03.2018 r.

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., **Bożena Bukowska**, Jaromir Michałowicz. 2015. *Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes.* Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 176-177: 62-70.

Oświadczam, że mój wkład w powstawanie publikacji polegał na pomocy w zaplanowaniu części eksperymentów oraz pomocy merytorycznej w napisaniu pracy. Mój udział oceniam na **10%**.

B Bukowska

.....

Łódź, dn. 5.03.2018 r.

Dr hab. Jaromir Michałowicz, prof. nadzw. UŁ
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Bożena Bukowska., **Jaromir Michałowicz**. 2015. *Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes.* Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 176-177: 62-70.

Oświadczam, że mój wkład w powstawanie publikacji polegał na opracowaniu koncepcji pracy. Dotyczył on także pomocy w interpretacji uzyskanych wyników oraz nadaniu publikacji ostatecznej formy. Mój udział oceniam na **30%**.

J. Michałowicz

Łódź, dn. 5.03.2018 r.

Mgr Aneta Wolska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, Bożena Bukowska., Jaromir Michałowicz. 2016. *Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study)*. Journal of Hazardous Materials 307: 328-335.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Udział w pracy związany był także z wykonaniem części analiz dotyczących zmian w poziomie jonów wapnia w cytozolu komórek i zmian w procesie eksternalizacji fosfatydyloseryny w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Wykonałam także analizy zmian w aktywności kaspazy-3 i aktywności kalpain w badanych komórkach. Ponadto, dokonałam interpretacji części uzyskanych wyników i współuczestniczyłam w napisaniu publikacji. Mój udział oceniam na **50%**.

.....Aneta Wolska (Maćczak).....

Łódź, dn. 5.03.2018 r.

Mgr Monika Cyrkler
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., **Monika Cyrkler**, Bożena Bukowska., Jaromir Michałowicz. 2016. *Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study)*. Journal of Hazardous Materials 307: 328-335.

Oświadczam, że mój wkład w powstawanie publikacji polegał na wykonaniu części analiz dotyczących określenia zmian w poziomie jonów wapnia w cytozolu komórek i zmian w procesie eksternalizacji fosfatydyloseryny w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Mój udział oceniam na **10%**.

.....Monika Jaworowicz (Cyrkler).....

Łódź, dn. 5.03.2018 r.

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, **Bożena Bukowska.**, Jaromir Michałowicz. 2016. *Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study)*. Journal of Hazardous Materials 307: 328-335.

Oświadczam, że mój wkład w powstawanie publikacji związany był z pomocą w interpretacji uzyskanych wyników oraz pomocą merytoryczną podczas pisania artykułu. Mój udział oceniam na **10%**.

B Bukowska

.....

Łódź, dn. 5.03.2018 r.

Dr hab. Jaromir Michałowicz, prof. nadzw. UŁ
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, Bożena Bukowska., **Jaromir Michałowicz**. 2016. *Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study)*. Journal of Hazardous Materials 307: 328-335.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu większości eksperymentów. Dotyczył on także pomocy w interpretacji uzyskanych wyników oraz pomocy w napisaniu publikacji. Mój udział oceniam na **30%**.

J. Michałowicz

Łódź, dn. 5.03.2018 r.

Mgr Aneta Wolska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, Bożena Bukowska., Jaromir Michałowicz. 2017. *Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study)*. Toxicology in Vitro 41: 143-149.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w tworzeniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Udział w pracy związany był z wykonaniem części analiz dotyczących zmian w poziomie reaktywnych form tlenu (RFT) oraz wysoce reaktywnych form tlenu w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Wykonałam również analizy zmian w procesie peroksydacji lipidów, poziomie zredukowanego glutationu (GSH) oraz aktywności enzymów antyoksydacyjnych (SOD, GSH-Px, CAT) w badanych komórkach. Ponadto, dokonałam interpretacji części uzyskanych wyników i uczestniczyłam w napisaniu publikacji. Mój udział oceniam na **55%**.

Aneta Wolska (Maćczak)

Łódź, dn. 5.03.2018 r.

Mgr Monika Cyrkler
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., **Monika Cyrkler**, Bożena Bukowska., Jaromir Michałowicz. 2017. *Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study)*. Toxicology in Vitro 41: 143-149.

Oświadczam, że mój wkład w powstawanie publikacji polegał na wykonaniu części analiz dotyczących zmian w poziomie reaktywnych form tlenu (RFT) oraz wysoce reaktywnych form tlenu w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Mój udział oceniam na **10%**.

.....Monika Jaroszewicz (Cyrkler).....

Łódź, dn. 5.03.2018 r.

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

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Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, **Bożena Bukowska.**, Jaromir Michałowicz. 2017. *Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study)*. Toxicology in Vitro 41: 143-149.

Oświadczam, że mój wkład w powstawanie publikacji polegał na pomocy w interpretacji uzyskanych wyników oraz pomocy merytorycznej podczas pisania pracy. Mój udział oceniam na **10%**.

B. Bukowska

.....

Łódź, dn. 5.03.2018 r.

Dr hab. Jaromir Michałowicz, prof. nadzw. UŁ
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

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Dotyczy publikacji:

Aneta Maćczak., Monika Cyrkler, Bożena Bukowska., **Jaromir Michałowicz**. 2017. *Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (in vitro study)*. Toxicology in Vitro 41: 143-149.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w tworzeniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Dotyczył on również pomocy w interpretacji uzyskanych wyników oraz pomocy w napisaniu publikacji. Mój udział oceniam na **25%**.

J. Michałowicz

Łódź, dn. 5.03.2018 r.

Mgr Aneta Wolska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Piotr Duchnowicz, Paulina Sicińska, Maria Koter-Michalak, Bożena Bukowska, Jaromir Michałowicz. 2017. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities.* Food and Chemical Toxicology 110: 351-359.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Udział w pracy związany był z przygotowaniem próbek do pomiarów z użyciem elektronowego rezonansu paramagnetycznego (EPR) dotyczących analiz płynności błony, mikrolepkości wnętrza erytrocytu oraz zmian konformacyjnych białek błonowych pod wpływem bisfenolu A i jego analogów. Dokonałam również analizy zmian w oporności osmotycznej, poziomie wewnątrzkomórkowego ATP oraz uszkodzeń oksydacyjnych białek w ludzkich erytrocytach. Ponadto wykonałam pomiary dotyczące poziomu grup tiolowych oraz aktywności acetylocholinoesterazy (AChE) i ATPazy Na^+/K^+. Mój udział w pracy obejmował także interpretację części uzyskanych wyników oraz częściowe napisanie pracy. Mój udział oceniam na **50%**.

....Aneta...Wolska...(Maćczak.).....

Łódź, dn. 5.03.2018 r.

Dr hab. Piotr Duchnowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

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Aneta Maćczak., **Piotr Duchnowicz**, Paulina Sicińska, Maria Koter-Michalak, Bożena Bukowska., Jaromir Michałowicz. 2017. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities.* Food and Chemical Toxicology 110: 351-359.

Oświadczam, że mój wkład w powstawanie publikacji polegał na wykonaniu pomiarów z użyciem elektronowego rezonansu paramagnetycznego (EPR) dotyczących analiz płynności błony, mikrolepkości wnętrza erytrocytu oraz zmian konformacyjnych białek błonowych w badanych komórkach pod wpływem bisfenolu A i jego analogów. Dotyczył on również pomocy w interpretacji uzyskanych wyników. Mój udział oceniam na **10%**.

.....

Łódź, dn. 5.03.2018 r.

Dr Paulina Sicińska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Piotr Duchnowicz, **Paulina Sicińska**, Maria Koter-Michalak, Bożena Bukowska., Jaromir Michałowicz. 2017. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities.* Food and Chemical Toxicology 110: 351-359.

Oświadczam, że mój wkład w powstawanie publikacji polegał na pomocy merytorycznej związanej z wykonywaniem analiz aktywności ATPazy Na^+/K^+ w erytrocytach człowieka pod wpływem bisfenolu A i jego analogów. Mój udział oceniam na **5%**.

Paulina Sicińska

Łódź, dn. 5.03.2018 r.

Prof. dr hab. Maria Koter-Michalak
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Piotr Duchnowicz, Paulina Sicińska, Maria Koter-Michalak, Bożena Bukowska., Jaromir Michałowicz. 2017. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na+/K+ ATPase and AChE activities.* Food and Chemical Toxicology 110: 351-359.

Oświadczam, że mój wkład w powstawanie publikacji polegał na współudziale w zaplanowaniu części eksperymentów. Mój udział oceniam na **5%**.

Maria Koter

Łódź, dn. 5.03.2018 r.

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Dotyczy publikacji:

Aneta Maćczak., Piotr Duchnowicz, Paulina Sicińska, Maria Koter-Michalak, **Bożena Bukowska.**, Jaromir Michałowicz. 2017. *The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocytes membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities.* Food and Chemical Toxicology 110: 351-359.

Oświadczam, że mój wkład w powstawanie publikacji polegał na pomocy w interpretacji uzyskanych wyników oraz pomocy merytorycznej podczas pisania pracy. Mój udział oceniam na **5%.**

B Bukowska

Łódź, dn. 5.03.2018 r.

Dr hab. Jaromir Michałowicz, prof. nadzw. UŁ
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

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J. Michałowicz

7. KOPIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

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Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes

Aneta Maćczak, Bożena Bukowska, Jaromir Michałowicz *

Department of Environmental Pollution Biophysics, Faculty of Biology and Environmental Protection, University of Łódź, Pomorska 141/143 St., 90-001 Łódź, Poland

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ABSTRACT

Bisphenol A (BPA) has been shown to provoke many deleterious impacts on human health, and thus it is now successively substituted by BPA analogues, whose effects have been poorly investigated. Up to now, only one study has been realized to assess the effect of BPA on human erythrocytes, which showed its significant hemolytic and oxidative potential. Moreover, no study has been conducted to evaluate the effect of BPA analogues on red blood cells. The purpose of the present study was to compare the impact of BPA and its selected analogues such as bisphenol F (BPF), bisphenol S (BPS) and bisphenol AF (BPAF) on hemolytic and morphological changes and hemoglobin oxidation (methemoglobin formation) of human erythrocytes. The erythrocytes were incubated with different bisphenols concentrations ranging from 0.5 to 500 μg/ml for 1, 4 and 24 h. The compounds examined caused hemolysis in human erythrocytes with BPAF exhibiting the strongest effect. All bisphenols examined caused methemoglobin formation with BPA inducing the strongest oxidative potential. Flow cytometry analysis showed that all bisphenols (excluding BPS) induced significant changes in erythrocytes size. Changes in red blood cells shape were conducted using phase contrast microscopy. It was noticed that BPA and BPAF induced echinocytosis, BPF caused stomatocytosis, while BPS did not provoke significant changes in shape of red blood cells. Generally, the results showed that BPS, which is the main substituent of bisphenol A in polymers and thermal paper production, exhibited significantly lower disturbance of erythrocyte functions than BPA.

1. Introduction

Bisphenols are chemical compounds that are used in huge amounts in the manufacturing of polycarbonates, epoxy resins and thermal paper (Inoue et al., 2008). They are widely represented by bisphenol A (BPA) and its analogues, e.g. bisphenol F (BPF), bisphenol S (BPS) and bisphenol AF (BPAF) (Liao and Kannan, 2013). Bisphenols are present in plastic containers, bottles, medical materials, toys, varnishes, newspapers, books, faxes and other products (Liao et al., 2012a). Among bisphenols, BPA and BPF are produced in the highest amounts. Global production of BPA was 6.8 million tons in 2013 (GrandViewResearch, 2014). Recently, the production of other bisphenols such as BPS and BPF has also significantly increased, which is associated with replacement of BPA with those substances in the synthesis of polymers and thermal paper (Vandenberg et al., 2007; Lotti et al., 2013). For a couple of years, the use of BPA in the production of plastic infant feeding bottles and some other consumers products has been restricted in the European Union and the United States (Huang et al., 2012; Liao and Kannan, 2013), while the use of BPS, BPF and BPAF is presently not regulated, and thus those compounds are used without any restriction (Eladak et al., 2015).

Bisphenols have been repeatedly determined in human body. BPA has been detected in the urine of the USA population in the concentrations ranging from 0.4 to 149 ng/ml (Calafat et al., 2008). In Canadian population, Bushnik et al. (2010) determined BPA (mean 1.16 μg/dm^3) in 90.7% of urine samples, while Liao et al. (2012b) detected BPS in 81% of urine samples (from trace to 21 μg/dm^3) of the populations of the USA, China, India and other Asian countries. In occupational survey, significant mean BPA concentration (mean—approximately 5 μg/ml; 5.4 μg/g creatinine) was determined in the urine of Chinese workers employed in the production of epoxy resins (He et al., 2009).

Toxicological studies have mainly focused on BPA. They have shown toxic, endocrine, mutagenic and carcinogenic effects exerted by BPA both on in vitro and in vivo models (Vandenberg et al., 2007). BPA as an endocrine disruptor has been shown to bind to estrogen and androgen receptors (Huang et al., 2012). BPA has also been supposed to increase risk of obesity, diabetes and heart disease (Michałowicz, 2014).

The replacement of BPA with other bisphenols like BPS and BPF in the production of baby bottles and food and water containers led to the increase of the exposure of human population to these substances. Nevertheless, a small number of studies concerning toxic effects of

* Corresponding author. Tel./fax: +48 426354453 73.
E-mail address: michalow@biol.uni.lodz.pl (J. Michałowicz).

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1532-0456/

BPA analogues makes impossible to state whether those substituents are safe for human health.

Erythrocytes are the most plentiful cells of blood participating in numerous key biochemical and physiological functions. Numerous xenobiotics are transported in blood, and thus the erythrocytes are significantly exposed to their action (Stasiuk et al., 2009).

Phenolic substances have been proven to provoke morphological and oxidative changes in red blood cells. Bukowska et al. (2009) noted that various phenols, and particularly 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) changed erythrocyte membrane fluidity and altered the contents of erythrocyte membrane proteins, which led to morphological changes in red blood cells. In another studies, it was proven that phenol and 2,4-DCP induced hemolysis of red blood cells (Duchnowicz et al., 2002; Bukowska and Kowalska, 2004). Phenols have also been shown to oxidize hemoglobin. Duchnowicz et al. (2002) revealed that chlorophenols and particularly 2,4-dimethylphenol caused methemoglobin formation in human erythrocytes (Duchnowicz et al., 2002).

It is known that increased methemoglobin level is associated with formation of deposits of denatured hemoglobin (Heinz bodies), which change erythrocyte morphology, and then cause premature removal of red blood cells from circulation (Giardina et al., 1995; Bartosz, 2009).

According to our best knowledge, up to now, only one study has been conducted to analyze the effect of BPA on human erythrocytes, which showed that BPA exhibited hemolytic and oxidative potential in this cell type. Moreover, no study has been performed to assess the effect of BPA analogues on red blood cells. That is why, the aim of this study was to compare the effect of BPA and its selected analogues such as BPF, BPS and BPAF (Fig. 1) on hemolytic and morphological changes and hemoglobin oxidation in human erythrocytes.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99%, 2,2-bis(4-hydroxyphenyl)propane) (BPA), bisphenol F (99%, 4,4′-dihydroxydiphenylmethane) (BPF), bisphenol S (99%, 4,4′-sulfonyldiphenol) (BPS), bisphenol AF (99%, 2,2-bis(4-hydroxyphenyl)hexafluoropropane) (BPAF) were purchased from Sigma-Aldrich, USA. Other chemicals were bought in POCH (Poland). Bisphenols were dissolved in ethanol. The final concentration of ethanol in the samples was 0.5%. We compared changes in all parameters studied between the samples containing the erythrocytes with Ringer buffer and samples consisting the erythrocytes with Ringer buffer and ethanol. Because we have not found any statistically significant differences, we considered that the concentration of ethanol used in the experiments was not toxic for the cells studied.

2.2. Erythrocyte isolation

Leucocyte buffy-coat was obtained from blood collected in Blood Bank in Łódź, Poland. Blood was obtained from 20 healthy volunteers (aged 18–50), who showed no signs of infection disease symptoms at the time the blood samples were collected. The leucocyte-buffy coat was centrifuged (3000 rpm for 10 min at 4 °C) and washed twice with Ringer buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 32 mM HEPES, 25 mM Tris, pH = 7.4). The isolated erythrocytes of 5% hematocrit suspended in Ringer buffer were incubated with bisphenols (0.5–500 μg/ml) at 37 °C for 1 h, 4 h and 24 h in the total darkness. The control samples contained the erythrocytes incubated with Ringer buffer and ethanol (final concentration of ethanol was 0.5%).

2.3. Hemolysis

The measurement of the content of released hemoglobin from the erythrocytes was used as the parameter of cell viability (Drabkin, 1946). After incubation, the erythrocytes were centrifuged at 3000 rpm for 10 min at 4 °C and resuspended in Ringer buffer. The absorbance of hemoglobin in the supernatant was determined on the basis of the absorbance measurement at a wavelength of 542 nm (Specord 250 plus, Analytik Jena AG). Percent of hemolysis of the erythrocytes was calculated from the equation:

$$H[\%] = A_{pb}/A_{100\%}x100$$

where: H [%]—percent of hemolysis, A_{pb}—hemoglobin absorbance in the samples with the erythrocytes incubated with Ringer buffer or individual bisphenol, $A_{100\%}$—hemoglobin absorbance in the sample after complete hemolysis of the erythrocytes with water (100%), the samples after complete hemolysis were diluted with water (1:10) before measurement.

2.4. Hemoglobin oxidation

After incubation, the erythrocytes were lysed with distilled water, and the samples were centrifuged (12,000 rpm for 10 min at 4 °C). The percent of methemoglobin (met-Hb) in the total Hb content was calculated from the absorbance at wavelengths of 630 and 700 nm (BioTek ELx808, Bio-Tek) both for hemoglobin in control erythrocytes and hemoglobin in the erythrocytes treated with individual bisphenol. Positive control contained hemoglobin treated with potassium ferricyanide (100% met-Hb). In order to obtain positive control, hemoglobin was treated with

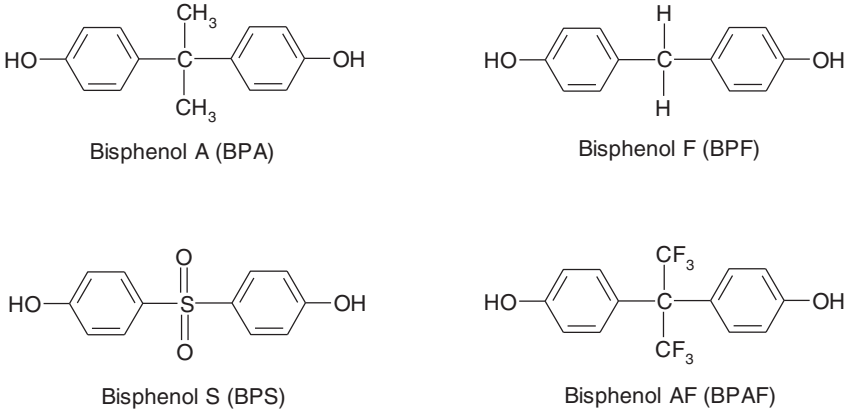

Fig. 1. Chemical structures of BPA and its analogues.

excess of potassium ferricyanide (1 M Hb Fe^{2+}: 3 M $K_4[Fe(CN)_6]$). Percent of met-Hb was calculated from the equation:

$$\text{Met-Hb}[\%] = \frac{(A_{630}-A_{700})}{(A_{100\%\ 630}-A_{100\%700})} \text{x}100\%$$

where: Met-Hb [%]—percent of methemoglobin, A_{630}—absorbance of the sample at 630 nm, A_{700}—absorbance of the sample at 700 nm, $A_{100\%metHb\ 630}$—absorbance of the sample at 630 nm treated with potassium ferricyanide, and $A_{100\%metHb\ 700}$—absorbance of the sample at 700 nm treated with potassium ferricyanide.

2.5. FSC parameter (erythrocyte size)

Flow cytometry (FCM) technique (LSR II Becton Dickinson) was used to assess the size of the erythrocytes. FMC gate on the erythrocytes has been established for data acquisition and cell size was determined with detection of low angle (FSC) light scattering. The data obtained were displayed in a form of a diagram of the cell number versus the light scatter and were analyzed by the standard computer program WinMDI 2.8. The light scattered near the forward direction (low angle) was expected to be proportional to the size (volume) of the particle. (De Grooth et al., 1987). The data were recorded for a total of 10,000 events per sample.

2.6. Microscopic analysis (erythrocyte shape)

Erythrocytes after 4 h and 24 h incubation with bisphenols were diluted with Ringer buffer to 0.02% hematocrit, and then immediately placed into a Petri dish. The cells were observed using the phase contrast microscope (Olympus, Japan) at the magnification of 600 ×.

2.7. Statistical analysis

Data are expressed as mean ± SD. Multiply comparisons among group mean differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences were considered to be statistically significant when p value was less than 0.05. All statistical analyses were done using STATISTICA software (StatSoft, Inc, Tulusa, USA). The mean value was calculated from 4 to 6 individual experiments (4–6 donors), whereas for each individual, an experimental point was a mean value of at least 3 replications.

3. Results

3.1. Hemolysis

The compounds studied caused different hemolytic changes in human erythrocytes. Generally, it was shown that hemolysis of the erythrocytes increased along with increasing concentrations of bisphenols studied and incubation time. Statistically significant increase in hemolysis was observed after 1 h incubation of the erythrocytes with BPA and BPF at 500 μg/ml and BPAF at 250 μg/ml and 500 μg/ml (Fig. 2A). After 4 h incubation, BPA and BPF at 250 μg/ml and 500 μg/ml caused statistically significant increase in hemolysis, while BPAF in the concentrations ranging from 100 to 500 μg/ml provoked statistically significant increase in the parameter studied (Fig. 2B). BPS did not cause statistically significant increase in erythrocytes hemolysis after 1 h and 4 h incubation (Fig. 2A, B). Statistically significant increase in hemolysis was noted for all compounds studied after 24 h incubation. It was observed that BPA and BPF in the concentrations ranging from 50 to 250 μg/ml caused statistically significant increase in the parameter examined, whereas BPS induced hemolysis only at its highest concentration of 250 μg/ml. The strongest changes were noted for BPAF that from the concentration of 10 μg/ml caused hemolytic alterations in the incubated cells (Fig. 2C).

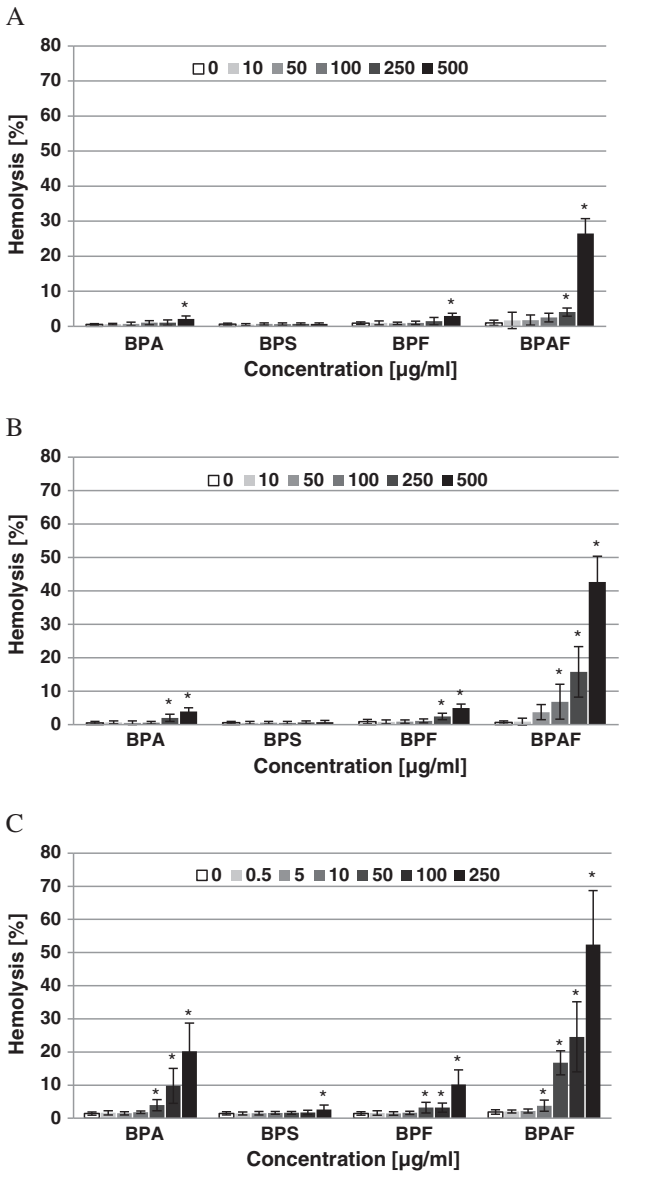

Fig. 2. Hemolytic changes in control erythrocytes and erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 10 to 500 μg/ml after 1 h (A) and 4 h (B) incubation, and from 0.5 to 250 μg/ml after 24 h incubation (C). (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

3.2. Hemoglobin oxidation

Statistically significant increase in methemoglobin level was induced by BPA and its analogues. After 1 h incubation, BPA, BPF and BPAF at 250 μg/ml and 500 μg/ml increased methemoglobin level (Fig. 3A). After 4 h incubation, BPA from the concentration of 100 μg/ml and BPF and BPAF from the concentration of 250 μg/ml increased hemoglobin oxidation (Fig. 3B). BPS did not cause statistically significant increase in the parameter studied both after 1 h and 4 h incubation (Fig. 3A, B). After 24 h incubation, all compounds examined from the concentration of 10 μg/ml caused statistically significant increase in met-Hb level but BPF and BPA in particular induced much stronger hemoglobin oxidation in comparison to BPAF, and particularly BPS (Fig. 3C).

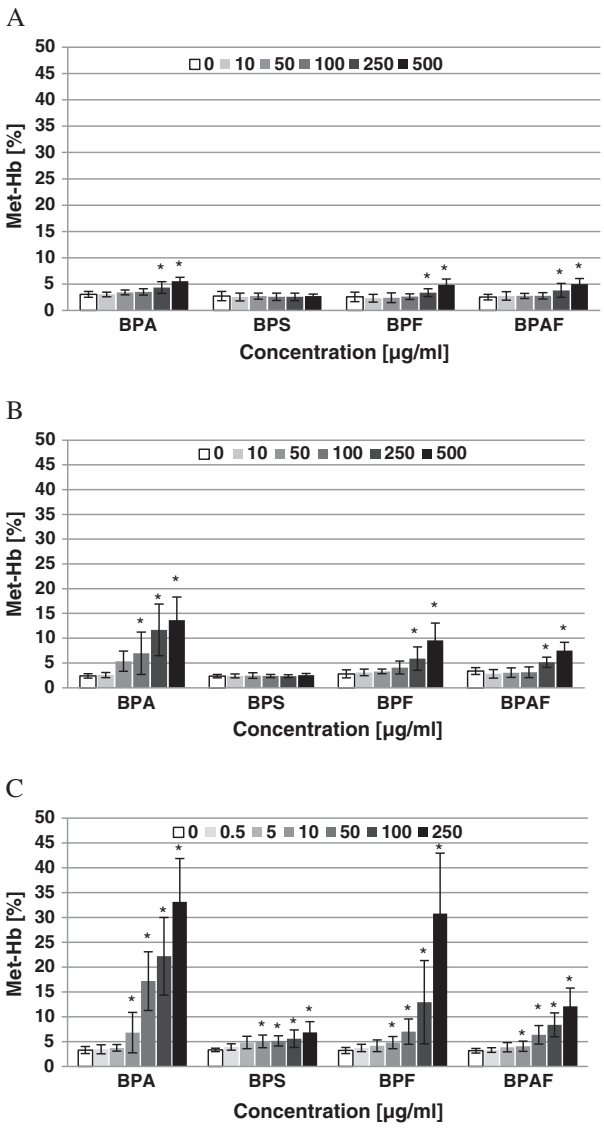

Fig. 3. Changes in methemoglobin level in control erythrocytes and erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 10 to 500 µg/ml after 1 h (A) and 4 h (B) incubation, and from 0.5 to 250 µg/ml after 24 h incubation (C). (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

3.3. Changes in the erythrocyte morphology

3.3.1. FSC parameter

Changes in FSC value may be associated with alterations in cell size. The laser light scattered near the forward direction of the particle (cell) is expected to be proportional to its size (volume). The study showed that BPA and most of its analogues induced significant changes in erythrocytes size. After 1 h incubation, an increase in FSC value was observed in the erythrocytes incubated with BPA, BPF and BPAF from the concentration of 500 µg/ml, 250 µg/ml and 100 µg/ml, respectively (Fig. 4A). After 4 h incubation, BPF, BPA and BPAF increased erythrocytes size from the concentration of 250 µg/ml, 100 µg/ml and 50 µg/ml, respectively (Fig. 4B), while BPS did not cause statistically significant changes in the parameter studied both after 1 and 4 h incubation (Fig. 4A, B). After 24 h incubation, BPA and BPF increased FSC parameter from the concentration of 100 µg/ml, whereas BPS caused statistically significant increase in cell size from the concentration of 250 µg/ml. The strongest changes in erythrocytes size were provoked by BPAF, which increased FSC value from the concentration of 25 µg/ml (Fig. 4C). Fig. 4D shows an increase in erythrocytes size (FSC value) treated with BPA at 100 µg/ml and 500 µg/ml after 4 h incubation.

3.3.2. Microscopic analysis

Microscopic analysis confirmed that BPA, BPF and BPAF in particular caused significant morphological changes in human erythrocytes, while BPS induced negligible alterations in this parameter. After 4 h and 24 h incubation, BPAF in the concentration of 25 µg/ml induced echinocyte formation. Moreover, BPAF at 100 µg/ml caused an increase in erythrocytes size, while at 250 µg/ml it provoked a decrease in this parameter as only echinocytes with shrinking size were observed (Fig. 5A). It was also noticed that BPS at 500 µg/ml even after 24 h incubation caused only slight deformation of erythrocytes (elongated cells); however no echinocyte and stomathocyte formation have been observed. After 24 h incubation, BPA at 100 µg/ml induced echinocyte formation, while at 250 µg/ml it decreased erythrocytes size because mainly echinocytes with shrinking size were observed. In contrast, BPF caused mainly stomathocyte formation (Fig. 5B).

Quantitative analysis of morphological changes in human erythrocytes was performed after 24 h incubation of the cells with BPA and its analogues in the concentrations ranging from 25 to 250 µg/ml. It was noted that BPAF caused the strongest effect. BPAF even at 25 µg/ml strongly altered erythrocyte morphology because it increased the parameter studied by 163.9% in comparison to control value (100%). BPA and BPF induced similar effects; however, BPF at 25 µg/ml induced substantially stronger changes in red blood cells morphology than BPA. It was also noticed that BPS did not provoke statistically significant changes in the parameter studied (Table 1).

4. Discussion

Extensive use of bisphenols caused increasing concern over the potential adverse effects provoked by these substances on human health (Song et al., 2014). The results of in vitro and in vivo studies as well as epidemiological surveys have demonstrated that BPA exhibits endocrine, hepatotoxic and neurotoxic potential and may increase risk of cancer development (Cabaton et al., 2006; Soto et al., 2013). Significantly less attention has been devoted BPA analogues including the effect of these substances on blood cells.

The aim of this study was to assess the effect of BPA and its selected analogues such as BPF, BPS and BPAF on hemolytic and morphological changes as well as hemoglobin oxidation in human erythrocytes.

Changes in hemolysis illustrate alterations in red blood cells viability. In this study, we observed that bisphenols caused different hemolytic changes in the erythrocytes, while BPAF induced the strongest alterations in this parameter. Moreover, it was noticed that hemolysis intensified along with the increase of the concentration of individual bisphenol and incubation time (Fig. 2)

Suthar et al. (2014) showed that BPA induced hemolysis in red blood cells. The authors suggested that BPA due to its lipophilic nature located in hydrophobic plasma membrane, and initiated free radical formation. They also claimed that BPA bound to hemoglobin iron, which led to dissociation of hemoglobin subunits and release of iron ions into cytoplasm. Because iron ion participates in the formation of highly reactive hydroxyl radical, damage to cellular membrane by initiating lipid peroxidation occurred, which resulted in hemolysis of red blood cells. In another study, Bukowska and Kowalska (2004) showed that phenol and catechol caused damage to erythrocytes, which led to hemolysis. Similarly, Boge and Roche (1996) observed hemolytic changes in red blood cells incubated with phenolic compounds like hydroquinone and catechol.

Hemolysis may proceed as a result of methemoglobin formation, which leads to damage to cellular membrane of the erythrocytes (Giardina et al., 1995) and subsequent excessive removal of red blood cells from circulation (Bartosz, 2009).

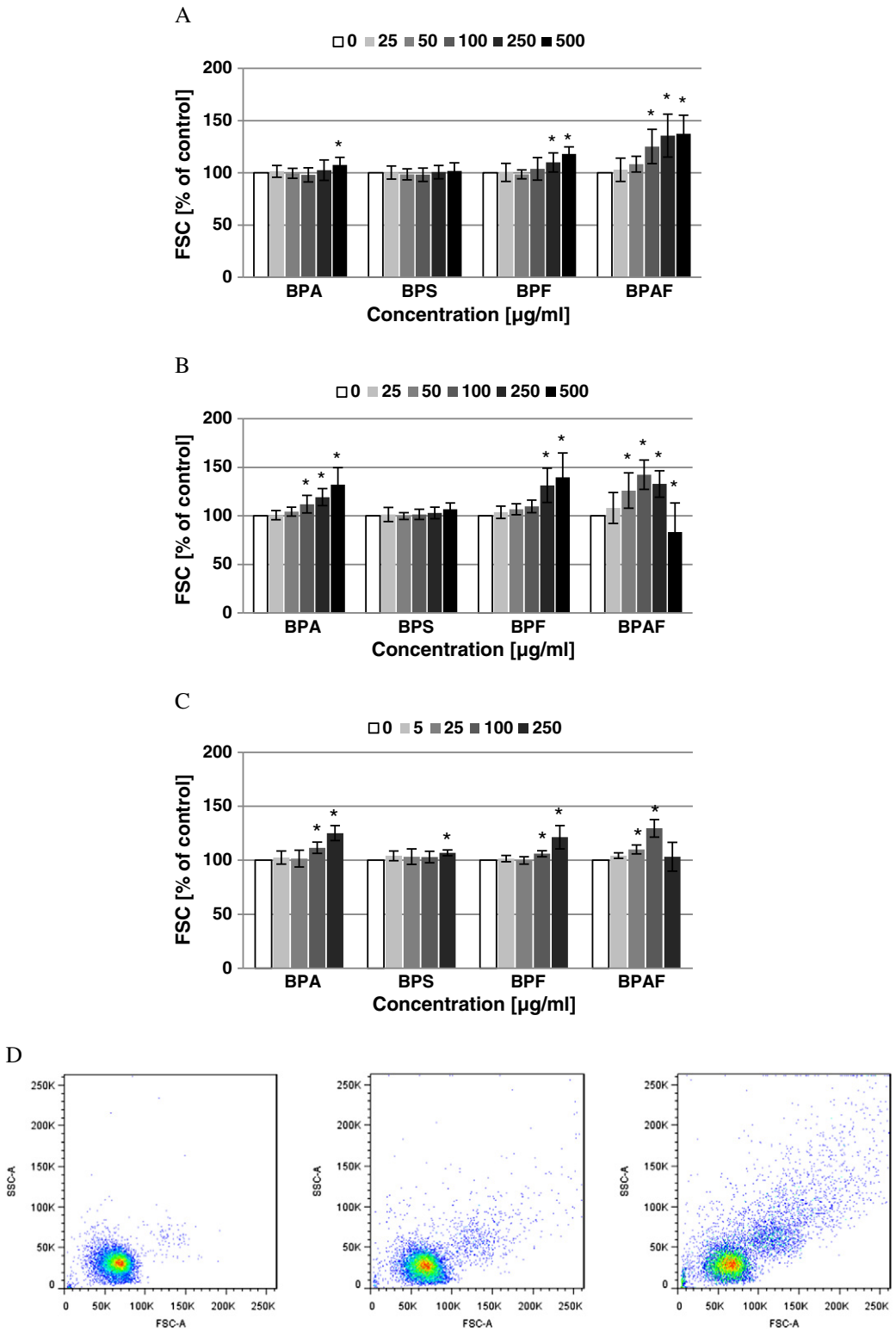

Fig. 4. Changes in FSC parameter (cell size) of control erythrocytes and erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 25 to 500 µg/ml after 1 h (A) and 4 h (B) incubation, and from 5 to 250 µg/ml after 24 h incubation. (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test. Quantification of changes in size of erythrocytes (FSC parameter) exposed to BPA. The analysis was performed by flow cytometry. Control cells, cells treated with BPA at 100 µg/ml, cells treated with BPA at 500 µg/ml for 4 h (D). The figures show changes in FSC parameter (cell size) (X axis) versus SSC parameter (cell shape) (Y axis).

In old or oxidatively damaged erythrocytes, where the reduction systems may be damaged or insufficiently active, met-Hb, instead of being readily reduced, can be slowly transformed into hemichromes that in turn may react with band 3 protein and/or spectrin, initiating some of the cross-linking processes that are known to occur in vitro. That is why, interaction of met-Hb with the plasma membrane is upstream of all the reactions that may lead to elimination of old and damaged erythrocytes (Low et al., 1985; Giardina et al., 1995).

Literature data have revealed that phenolic compounds exhibit oxidative potential mainly in respect to superoxide radical formation,

which affects heme iron oxidation, and thus methemoglobin formation (Bukowska and Kowalska, 2004).

In this study, we observed that bisphenols oxidized hemoglobin, while BPA exhibited the strongest oxidative potential (Fig. 3.). Other studies have demonstrated that BPA has oxidative potential. For instance, Bindhumol et al. (2003) showed that BPA induced oxidative stress in rat hepatocytes, whereas Lee et al. (2008) proved that BPA enhanced ROS formation in hippocampal neuronal cells of rats. Some studies have also revealed significant role of methyl group in protein (including hemoglobin) oxidation. Duchnowicz et al. (2002) compared the effect of various phenolic compounds like 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol and 2,4-dimethylphenol (2,4-DMP) on human erythrocytes and concluded that phenols with methyl groups can strongly oxidize hemoglobin. Moreover, Bukowska et al. (2007) observed that 2,4-DMP induced much stronger protein oxidation (increased carbonyl group content) in human erythrocytes in comparison to catechol and 2,4-DCP.

Human erythrocytes have characteristic biconcave shape, which provides higher surface to volume ratio, and thus enhances gas exchange and guarantees greater deformability. The proper shape of the erythrocyte determines its existence in circulatory system for about 120 days. Changes in erythrocytes size and shape induced by external factors usually lead to an accelerated elimination of these cells from circulation (Stasiuk et al., 2009).

In order to analyze size and shape of human erythrocytes, we used flow cytometry and phase microscope techniques. Our study showed that BPA, BPF and BPAF caused significant changes in erythrocyte morphology (Figs. 4, 5A, 5B). It was observed that BPAF induced the strongest changes in size and shape of the erythrocytes (Fig. 5A), while BPS caused negligible alterations in the parameters studied (Fig. 5B).

An increase in erythrocytes size is usually associated with damage to cellular membrane. We observed that changes in size of red blood cells accompanied hemolytic alteration, which may be the result of water influx into the cells and/or incorporation of xenobiotics into cellular membrane. It is also known that cytoskeletal proteins and integral membrane proteins are responsible for maintaining the erythrocyte shape. Various xenobiotics including phenols may damage to proteins of the erythrocytes (Bukowska et al., 2009; Bukowska et al., 2011), which results in the conversion of normal discocyte to echinocyte or stomathocyte (Stasiuk et al., 2009).

Microscopic examination of the erythrocytes showed changes in their cellular membrane, which are characteristic for echinocytes and stomatocytes. According to Sheetz and Singer (1974) hypothesis, changes in erythrocytic shape arise from differential expansion of two monolayers of membrane lipid bilayer. Thus, echinocytes are formed during insertion of xenobiotics into outer monolayer, whereas stomatocytes are formed as a result of xenobiotic accumulation in inner monolayer of the cellular membrane (Sheetz and Singer, 1974; Stasiuk et al., 2009). Numerous toxicants have been proven to exert significant effect on plasma membrane as the result of their interactions with its membrane components, i.e. lipids and proteins (Marczak et al., 2007; Bors et al., 2012; Reinhart et al., 2014). In our experiments, echinocytes appeared after red blood cell treatment with BPA and BPAF, while stomatocytes were formed after exposure of the cells to BPF (Fig. 4A, B). Quantitative analysis of morphological changes of erythrocytes showed that BPAF induced the strongest morphological alterations in the cells studied, while BPS did not induce statistically significant effect on this parameter (Table 1).

Bisphenols are a group of chemicals that share similar molecular structures containing two para-hydroxyphenyl functionalities, and thus they may exhibit similar toxicological effects. Nevertheless, due to various physicochemical properties (including hydrophobicity) of BPA and its analogues, which are determined by various functional groups (Song et al., 2014), they were able to exert different cytotoxic effects in the erythrocytes. Based on the result on this study, we observed

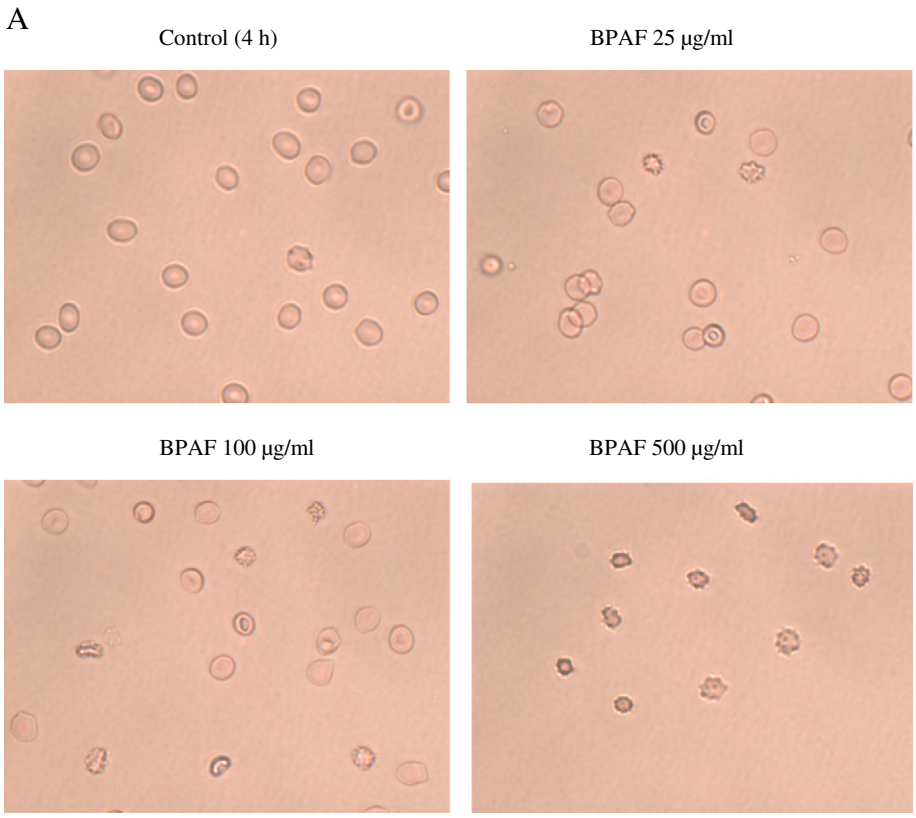

Fig. 5. Microscopic images showing alterations in shape of the erythrocytes incubated with BPAF at 25 μg/ml, 100 μg/ml and 250 μg/ml for 4 h (A), BPS at 500 μg/ml and BPA and BPF at 100 μg/ml and 250 μg/ml for 24 h (B).

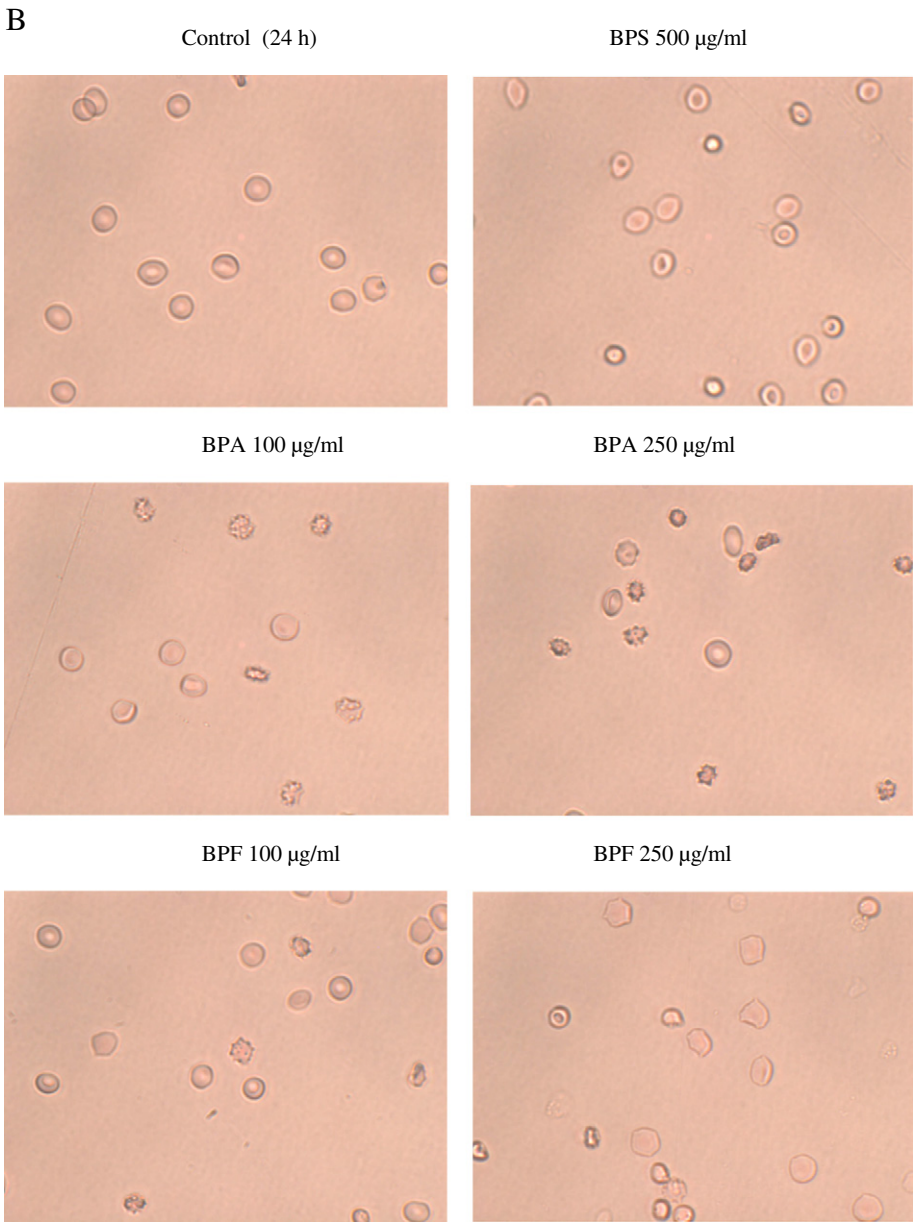

Fig. 5 (*continued*).

that cytotoxic potential of bisphenols studied was as follows: BPAF > BPA > BPF > BPS.

Hydrophobicity determines solubility of phenols in cellular fractions (Hansch et al., 2000). An increase in log $P_{o/w}$ value enhances the effectiveness of diffusion of xenobiotic across cellular membrane, which may influence stronger cytotoxicity of individual compound (Tsutsui et al., 1997).

It is highly probable that BPAF showed the most powerful cytotoxicity because of its strongest hydrophobic (lipophilic) properties among examined substances (evidenced by its highest log $P_{o/w}$ value equal to 4.47). The values of log $P_{o/w}$ for BPA and BPF are 3.34 and 2.91, respectively (NTP, 2008; Mohapatra et al., 2010), while BPS is characterized by the lowest log $P_{o/w}$ value of 2.15 (Mohapatra et al., 2010).

Other studies have also shown that BPA analogues exhibit significant toxicity. Grignard et al. (2011) and Cabaton et al. (2009) noticed that BPS and BPF exhibited similar to BPA estrogenic activities, while Tsutsui et al. (2000) noted that BPAF revealed stronger cytoxicity than BPA in Chinese hamster ovary cells.

It must be underlined that significant cytotoxicity of BPAF may be also related with reactivity of its CF_3 moieties. It has been proven that CF_3 group is much more electronegative and reactive than for example methyl group(s) (BPA), and thus it is able to create halogen bonds between BPAF and biological structures (Bronowska, 2011). The formation of halogen bonds between BPAF and oxygen atoms and carbonyl or

Table 1
Changes in shape of human erythrocytes treated with BPA, BPS, BPF or BPAF in the concentrations ranging from 25 to 250 µg/ml in comparison to control (100%) after 24 h incubation.

Compound concentration [µg/ml]	Percent of cells of changed shape			
	Control	25	100	250
BPA	100	113.0 ± 8.44	141.3 ± 11.85*	191.4 ± 28.02*
BPF	100	133.7 ± 22.46	147.7 ± 14.44*	197.1 ± 9.99*
BPS	100	104.4 ± 7.57	108.5 ± 14.76	105.4 ± 26.22
BPAF	100	163.9 ± 10.45*	196.7 ± 34.45*	233.9 ± 9.25*

* Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

hydroxyl residues changes molecule geometry, which results in more intense biological interactions (Bissantz et al., 2010; Zhou et al., 2010).

It must be noted that most of observed changes were provoked by relatively high bisphenols concentrations, which can influence human organism during subacute or acute poisoning. Nevertheless, some bisphenols were capable of inducing hemolytic and oxidative changes in the concentrations, which may possibly affect workers exposed to these compounds. BPAF at 10 μg/ml caused statistically significant increase in hemolysis of the cells studied. It was proven that the occurrence of excess of hemoglobin in serum may result in systemic or pulmonary hypertension, excessive platelet activation, and inflammation of blood vessels and kidney endothelium. The above described toxic effects are associated with direct cytotoxic and proinflammatory influence of released hemoglobin and heme as well as reaction of Hb with nitrogen oxide, which results in disturbances of endovascular homeostasis (Misztal and Tomasiak, 2011). Moreover, it was shown that BPA at 10 μg/ml caused statistically significant increase in met-Hb formation in the incubated cells. It has been proven that hemoglobin oxidation may lead to hypoxia or/and enhanced removal of the erythrocytes (as a result of Heinz bodies formation) from the body (Giardina et al., 1995).

5. Conclusions

a. BPA and its analogues provoked different changes in hemolysis, cell morphology and hemoglobin oxidation in human erythrocytes, which was due to different hydrophobicity and reactivity (the presence of various substituents) of these substances.
b. BPA exhibited the strongest oxidative potential, while BPAF revealed the most powerful cytotoxicity (hemolytic and morphological changes) in human erythrocytes.
c. BPS, which is the main substituent of bisphenol A in polymer synthesis and thermal paper production exhibited much lower toxicity in comparison to BPA in human erythrocytes .
d. The compounds examined after longer incubation time (24 h) induced hemoglobin oxidation (all bisphenols) and hemolytic changes (BPAF) in the concentrations that may possibly affect humans occupationally exposed to these substances or people accidentally poisoned with these compounds.

The authors declare that there is no conflict of interest.

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Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (*in vitro* study)

Aneta Maćczak, Monika Cyrkler, Bożena Bukowska, Jaromir Michałowicz*

Department of Environmental Pollution Biophysics, Faculty of Biology and Environmental Protection, University of Łódź, Pomorska 141/143 St., 90-001 Łódź, Poland

HIGHLIGHTS

- Bisphenol A (BPA), BPF, BPS and BPAF induced apoptotic changes in red blood cells.
- Changes in calcium ion level and phosphatidylserine translocation were noted.
- Caspase-3 and calpain were involved in suicidal erythrocyte death.
- BPAF was shown to exhibit the strongest apoptotic potential in red blood cells.
- BPS exhibited comparable eryptotic potential to BPA.

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ABSTRACT

Bisphenols are important chemicals that are widely used in the manufacturing of polycarbonates, epoxy resin and thermal paper, and thus the exposure of humans to these substances has been noted. The purpose of this study was to assess eryptotic changes in human erythrocytes exposed (*in vitro*) to bisphenol A (BPA) and its selected analogs, i.e.,bisphenol F (BPF), bisphenol S (BPS) and bisphenol AF (BPAF). The erythrocytes were incubated with compounds studied at concentrations ranging from 1 to 250 μg/mL for 4, 12 or 24 h. The results showed that BPA and its analogs increased cytosolic calcium ions level with the strongest effect noted for BPAF. It has also been revealed that all bisphenols analyzed, and BPAF and BPF in particular increased phosphatidylserine translocation in red blood cells, which confirmed that they exhibited eryptotic potential in this cell type. Furthermore, it was shown that BPA and its analogs caused significant increase in calpain and caspase-3 activities, while the strongest effect was noted for BPAF. BPS, which is the main substituent of bisphenol A in polymers and thermal paper production exhibited similar eryptotic potential to BPA. Eryptotic changes in human erythrocytes were provoked by bisphenols at concentrations, which may influence the human body during occupational exposure or subacute poisoning with these compounds.

1. Introduction

Bisphenols belong to the most widely used chemicals around the world with annual production for BPA reaching 5 million tons [1]. The exposure of human population to bisphenols is mainly associated with the use of numerous products made from polycarbonate plastics, epoxy resins and thermal paper [2,3]. Besides BPA, the worldwide production of BPA analogs such as bisphenol F (BPF), bisphenol S (BPS) and bisphenol AF (BPAF) has increased significantly [4], which is mainly due to a ban on BPA in some countries (including the European Union, since 2011) in various products including products for infants, packagings and bottles.

A lot of studies have reported BPA occurrence in the environment including air (200–17400 pg/m^3) [5], surface water (0.5–410 ng/L) and sludge (0.004–1.363 mg/kg) [6]. Food is the most important source of the general population exposure to bisphenols. The European Food Safety Authority estimated BPA dietary intake for adults and infants at up to 0.388 μg/kg and 0.875 μg/kg of body weight per day, respectively [7].

The presence of BPA in tap water (3.5–59.8 ng/dm^3) has also been noticed in some studies [8]. Moreover, humans are exposed to BPA and its analogs during dust inhalation and dermal contact with

* Corresponding author at: Department of Environmental Pollution Biophysics, Faculty of Biology and Environmental Protection, University of Łódź, Pomorska 141/143 St., Łódź 90-001, Poland. Fax: +48 426354473.
E-mail address: michalow@biol.uni.lodz.pl (J. Michałowicz).

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thermal paper. Liao et al. [9,10] determined bisphenols (mainly BPA, BPF and BPS) at concentrations ranging from 0.026 to 111 μg/g in dust in houses, offices and laboratories as well as detected high BPA and BPS concentrations (from trace to 22 mg/g) in numerous products made from thermal paper.

BPA has been repeatedly determined in human blood at concentrations ranging from trace amounts to 22.3 μg/L [2]. BPA has also been detected in the urine samples of humans environmentally and occupationally exposed. Calafat et al. [11] found BPA at concentrations ranging from 0.1 to 8.02 μg/L (mean 1.33 μg/L) in 95% of the 394 urine samples from adults in the USA, while in the urine of workers employed in the production of bisphenol A and epoxy resins, high mean BPA concentration (5 mg BPA/L; 5.4 mg BPA/g creatinine) was determined [12].

There are a lot of concerns about endocrine action of BPA [13,14] as well as hepatotoxic [15], neurotoxic [16] and carcinogenic [17] effects of this substance on the human body. BPA is also believed to provoke heart disease, type 2 diabetes, obesity and other disorders in humans [13,18–20]. The results of epidemiological and toxicological studies concerning the exposure and toxic action of BPF, and particularly BPS and BPAF are much more limited, which makes it necessary to assess the potential hazard of BPA analogs for human health.

Erythrocytes are the most plentiful cells in the circulatory system responsible for transporting oxygen and other chemicals, thus they are strongly exposed to xenobiotics like bisphenols. Up to now, only two studies have been conducted to assess the effect of bisphenol A (BPA) and its analogs on human erythrocytes [21,22]. Our previous study showed that BPA, BPS, BPF and BPAF exhibited different hemolytic and oxidative potential and caused morphological changes in red blood cells [23].

Eryptosis is the key process responsible for the removal of aged or damaged erythrocytes from circulation [23]. It has been proven that various xenobiotics can enhance eryptosis, which results in accelerated elimination of red blood cells from circulation, and may lead to anemia and other disorders development [23–25].

Taking the above into consideration we decided to compare the effect of BPA and its analogs like BPF, BPS and BPAF on selected eryptotic parameters of human erythrocytes. We have assessed changes in cytosolic calcium ions level, caspase-3 and calpain activities and phosphatidylserine translocation in red blood cells.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99%, 2,2-bis(4-hydroxyphenyl) propane) (BPA), bisphenol F (99%, 4,4′-dihydroxydiphenylmethane) (BPF), bisphenol S (99%, 4,4′-sulfonyldiphenol) (BPS), bisphenol AF (99%, 2,2-bis(4-hydroxyphenyl) hexafluoropropane) (BPAF) were purchased from Sigma–Aldrich, USA. Fluo-3/AM was bought from MoBiTec (Germany) and Annexin V-FITC kit was obtained from Becton Dickinson (USA). A kit for measuring caspase-3 activity (CaspGLOW) containing caspase-3 substrate (FITC-DEVD-FMK) and caspase-3 inhibitor (Z-VAD-FMK) was purchased from eBioscience (USA). A substrate for calpain analysis (CAMC, t-BOC-Leu-Met) was bought from Molecular Probes (USA). Calpain inhibitor (PD150606), ionomycin, betulinic acid, HBSS (Hanks' Balanced Salt Solution), HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) and other chemicals were purchased from Sigma–Aldrich (USA).

Bisphenols were dissolved in ethanol. The final concentration of ethanol in the samples was 0.2%. We compared changes in all parameters studied between the samples containing the erythrocytes with Ringer buffer and the samples containing the erythrocytes with Ringer buffer and ethanol. Because we did not find any statistically significant differences between the samples analyzed, we concluded that the concentration of ethanol used in the experiments was not toxic to the cells studied. The concentrations of BPA and its analogs used in this study did not induce significant hemolysis (excluding BPAF at 250 μg/mL) [22].

2.2. Erythrocytes isolation

A leukocyte buffy-coat was obtained from blood collected in the Blood Bank in Łódź, Poland. Blood was collected from 25 healthy volunteers (aged 18–55), who showed no signs of infectious disease symptoms at the time the blood samples were collected. The leukocyte buffy-coat was dilluted with PBS, centrifuged (3000 rpm for 10 min at 4 °C) and washed twice with Ringer buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 32 mM HEPES, 25 mM Tris, pH 7.4). The erythrocytes of 5% hematocrit were suspended in Ringer buffer, and then incubated with bisphenols (1–250 μg/mL) at 37 °C for 4 h, 12 h or 24 h in total darkness. Negative control samples contained the erythrocytes incubated with Ringer buffer and ethanol (final concentration 0.2%).

2.3. Cytosolic calcium ions level

The level of cytosolic calcium ions was analyzed by flow cytometry (LSR II, Becton Dickinson) using a fluorescent probe Fluo-3/AM according to manufacturer's protocol. Fluo-3/AM passes through membrane of living cells, in which it is cleavaged by intracellular esterases to Fluo-3. Fluo-3 exhibits green fluorescence after complexation with calcium ions. The intensity of Fluo-3 fluorescence was measured at 525 nm after excitation at 488 nm. The erythrocytes were treated with BPA, BPF, BPS or BPAF and incubated for 4 h or 12 h at 37 °C in total darkness. After incubation, the cells were centrifuged at 300 × *g* for 5 min at 4 °C and diluted with Ringer buffer (density 1×10^6 cells/mL). The cells were centrifuged (300 × *g* for 5 min at 4 °C), suspended in Fluo-3/AM solution (1 μM) and incubated for 20 min at 37 °C in total darkness. Then, HBSS (with 1% of BSA) was added to the cells suspension, which was incubated for 40 min at 37 °C in total darkness. The cells were centrifuged at 300 × *g* for 5 min at 4 °C, and washed twice with the HEPES buffer. After centrifugation, the cells were suspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. FMC gate on the erythrocytes has been established for data acquisition and the data were recorded for a total of 10,000 events per sample. Calcium ionomycin (1 μM) was used as a positive control. Calcium ionomycin is a polyether antibiotic produced by *Streptomyces conglobatus*. It is an effective mobile Ca^{2+} carrier, and is commonly used in the studies of Ca^{2+} transport across biological membranes.

2.4. FITC-conjugated Annexin V staining

During apoptosis, phosphatidylserine (PS) present in the internal layer of the plasma membrane moves to the external layer. Annexin V has high affinity for cells expressing PS on their surface. The externalization of phosphatidylserine was measured according to the procedure given by the manufacturer for Annexin V-FITC apoptosis detection kit. The cells were treated with BPA, BPF, BPS or BPAF and incubated for 24 h at 37 °C in total darkness. After incubation, the cells were centrifuged at 300 × *g* for 5 min at 4 °C and diluted with Ringer buffer (density 1×10^6 cells/mL). The cells were stained with Annexin V-FITC (1 μM) in Annexin V-binding buffer for 15 min at room temperature in total darkness. Flow cytometric analysis (LSR II, Becton Dickinson) was performed at excitation/emission of 488 nm/525 nm to visualize FITC fluorescence. FMC gate on the erythrocytes has been established for data acquisition and the data were recorded for a total of 10,000 events

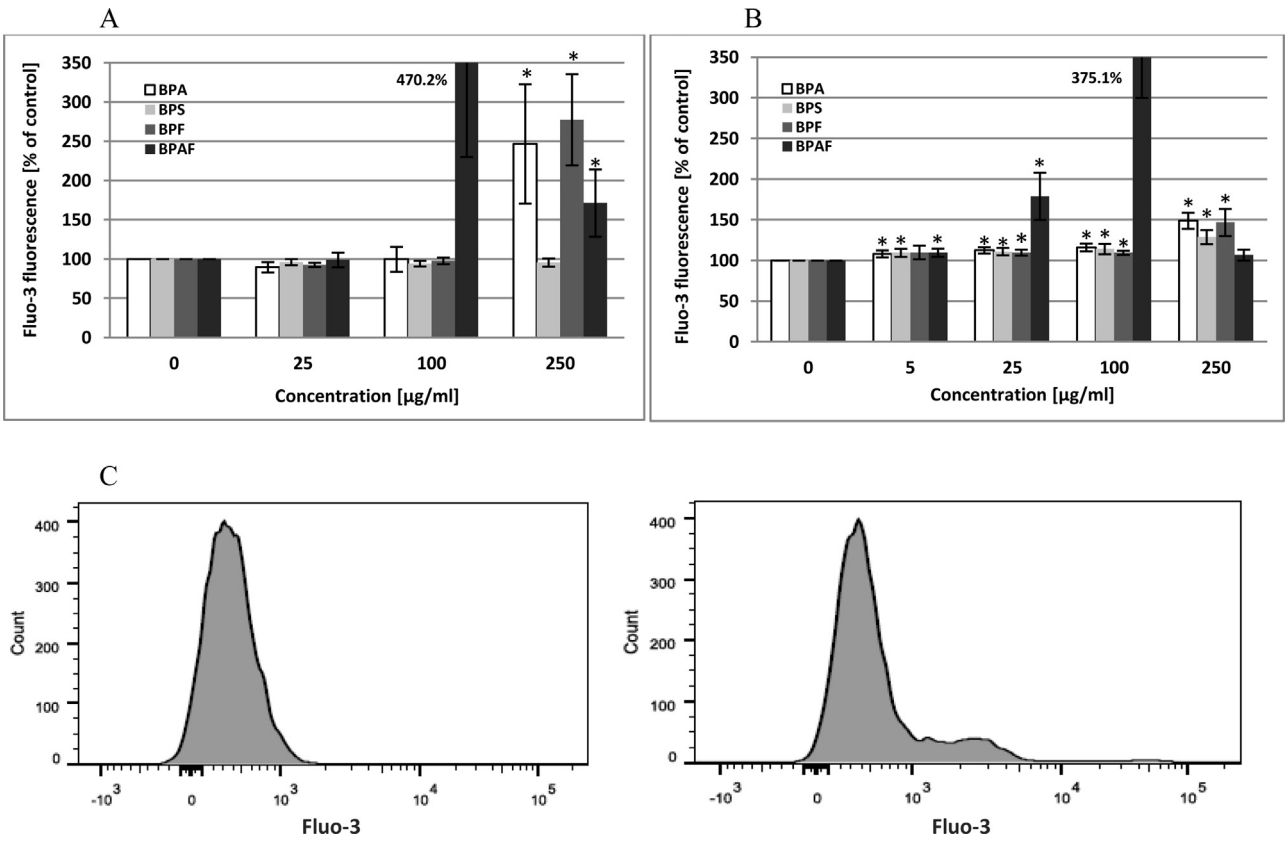

Fig. 1. Changes in calcium ions level of control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 25 to 250 μg/mL for 4 h (A) and from 5 to 250 μg/mL for 12 h (B), $n = 4$. *Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.
Quantification of changes in fluo-3 fluorescence (calcium ions level) in human erythrocytes exposed to BPAF. The analysis was performed by flow cytometry. Control cells, and cells treated with BPAF at 5 μg/mL for 12 h (C).

per sample. Betulinic acid (10 μM) was used as positive control. Betulinic acid is a pentacyclic lupane-type triterpene derivative of betulin isolated from the bark of *Betula pubescens*. It has been shown to induce *in vitro* eryptosis in human red blood cells through membrane permeabilization and phosphatidylserine translocation.

2.5. Caspase-3 activity

Caspase-3 activity was analyzed using flow cytometry (LSR II, Becton Dickinson). The analysis was conducted according to the manufacturer's protocol. The erythrocytes were treated with BPA, BPF, BPS or BPAF and incubated for 24 h at 37 °C in total darkness. After incubation, red blood cells were centrifuged at $300 \times g$ for 5 min at 4 °C and diluted with Ringer buffer (density 1×10^6 cells/mL). The cells were treated with cell permeable caspase-3 substrate (FITC-DEVD-FMK) at 2 μg/mL, which irreversibly binds to activated caspase-3, and incubated for 60 min at 37 °C in total darkness. Then, the cells were centrifuged at $300 \times g$ for 5 min at 4 °C, washed in wash buffer and analyzed with excitation/emission wavelengths of 495 nm and 525 nm, respectively. FMC gate on the erythrocytes has been established for data acquisition and the data were recorded for a total of 10,000 events per sample. In the experiment, betulinic acid (10 μM) was used as a positive control. Preincubation with caspase-3 inhibitor (Z-VAD-FMK) at 1 μL/mL was also conducted in order to measure the non-specific hydrolysis of the substrate.

2.6. Calpain activity

Calpain activity was analyzed using flow cytometry (LSR II, Becton Dickinson). The erythrocytes were treated with BPA, BPF, BPS or BPAF and incubated for 24 h at 37 °C in total darkness. After incubation, red blood cells were centrifuged at $300 \times g$ for 5 min at 4 °C and diluted with Ringer buffer (density 1×10^6 cells/mL). The cells were treated with cell permeable calpain substrate (t-butoxycarbonyl-Leu-Met) conjugated with 7-amino-4-chloromethylcoumarin (CMAC) at 10 μM, and incubated for 60 min at 37 °C in total darkness. The cells were centrifuged and washed with Ringer buffer. The analysis for 10,000 cells was performed at excitation/emission wavelengths of 355 nm and 450 nm, respectively. In the experiment, betulinic acid (10 μM) was used as a positive control. Preincubation with a calpain inhibitor (PD150606) at 15 μM was conducted in order to measure the non-specific hydrolysis of the substrate.

2.7. Statistical analysis

Data are expressed as mean ± SD. Multiple comparisons among group mean differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences were considered to be statistically significant when the p value was less than 0.05. All statistical analyses were done using STATISTICA software (StatSoft, Inc., Tulusa, USA). The mean value was calculated from 4 to 6 individual experiments (4–6 donors), whereas for each

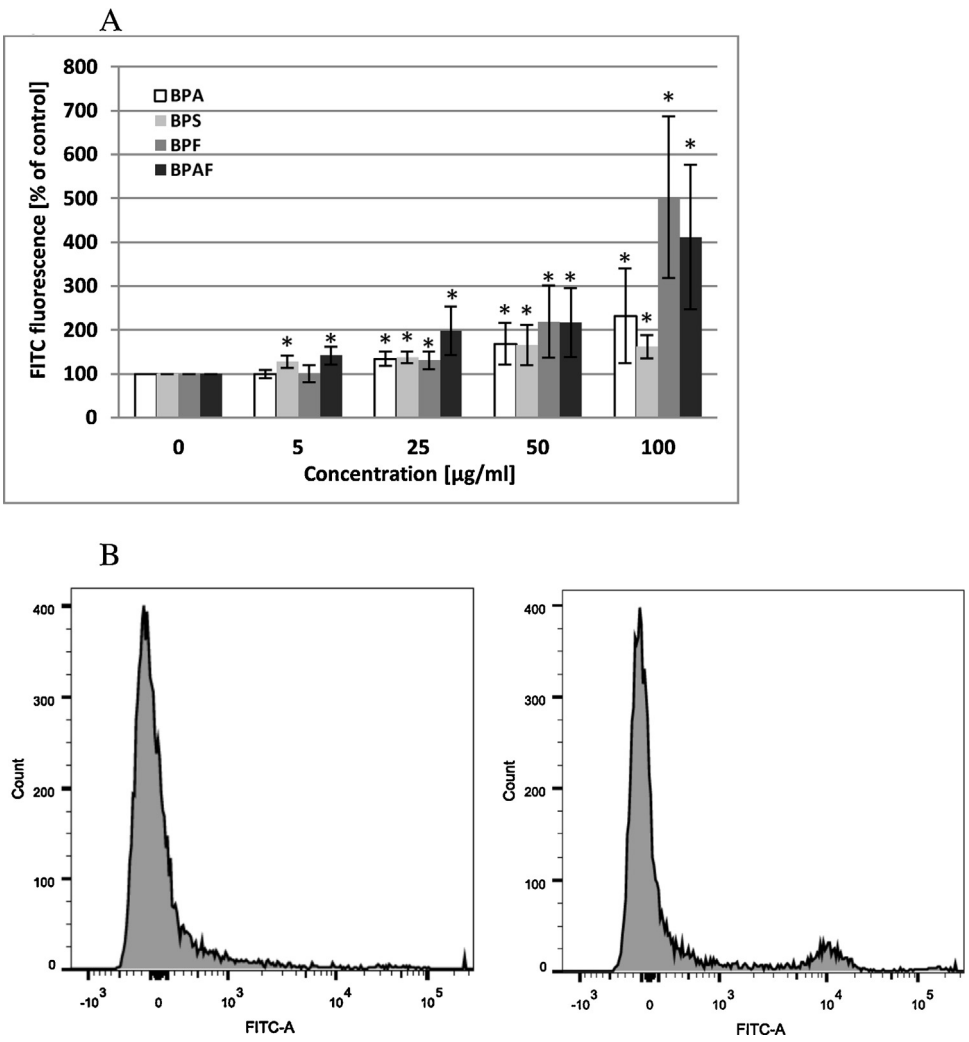

Fig. 2. Changes in phosphatidylserine exposure of control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 5 to 100 μg/mL for 24 h (A), $n = 5$. *Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.
Quantification of changes in FITC fluorescence (Annexin V binding) in human erythrocytes exposed to BPF. The analysis was performed by flow cytometry. Control cells, and cells treated with BPF at 25 μg/mL (B).

individual, an experimental point was a mean value of at least 2 replications.

3. Results

3.1. Cytosolic calcium ions level

The study showed that BPA and its analogs caused changes in cytosolic calcium ion levels in human erythrocytes. After 4 h incubation, a statistically significant increase in Ca^{2+} levels was noted in red blood cells treated with BPA and BPF at 250 μg/mL. It was also observed that BPAF increased Ca^{2+} levels at 100 μg/mL and 250 μg/mL, while BPS did not change the parameter studied (Fig. 1A). After 12 h incubation, BPA and its analogs at much lower concentrations caused an increase in calcium ion levels when compared with changes in the samples incubated for 4 h. An increase in Ca^{2+} level was observed in cells incubated with BPA (108.1–148.8%), BPS (109.5–128.7%) and BPF (109.8–146.7%) at concentrations ranging from 5 to 250 μg/mL (Fig. 1B). BPAF caused the strongest increase in the parameter studied at 100 μg/mL (375.1%), but at 250 μg/mL it depleted the Ca^{2+} level in human erythrocytes. Previous studies showed that BPAF exhibited significant hemolytic potential at 250 μg/mL [22], which probably contributed to efflux of Fluo-3 from the erythrocytes. Fig. 1C shows a sample histogram of Fluo-3 fluorescence in the erythrocytes following BPAF (5 μg/mL) exposure for 12 h.

3.2. Phosphatidylserine (PS) translocation

After 24 h incubation, BPA and its analogs caused a statistically significant increase in phosphatidylserine translocation in human erythrocytes. An increase in PS externalization was noted in cells incubated with BPA (134.2–232.5%), BPS (138.1–161.8%), BPF (131.1–503.1%) and BPAF (198.8–412.0%) at concentrations ranging from 25 to 100 μg/mL. Moreover, a statistically significant increase in the parameter studied was observed in cells treated with BPS (127.7%) and BPAF (141.8%) at 5 μg/mL. Among the compounds examined, BPAF at 5 μg/mL and 25 μg/mL induced the strongest changes, while BPF exhibited the most powerful effect at 100 μg/mL (Fig. 2A). Fig. 2B presents a sample histogram of Annexin V binding of erythrocytes (FITC fluorescence) following exposure to BPF (25 μg/mL) for 24 h.

3.3. Caspase-3 activity

For analysis of caspase and calpain activities, we have selected bisphenols concentrations that induced phosphatidylserine translocation in the cells studied. BPA and its analogs activated

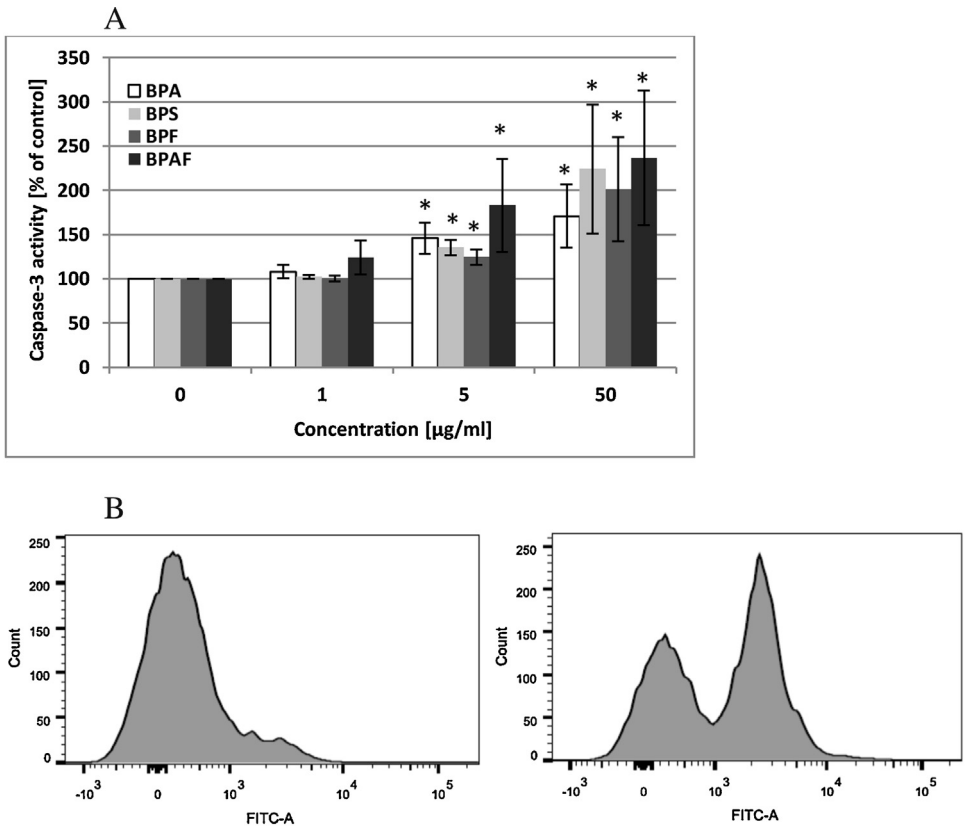

Fig. 3. Changes in caspase-3 activity of control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 1 to 50 μg/mL for 24 h (A), $n = 4$. *Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.
Quantification of changes in FITC fluorescence (caspase-3 activity) in human erythrocytes exposed to BPS. The analysis was performed by flow cytometry. Control cells, and cells treated with BPS at 50 μg/mL (B).

caspase-3 in human erythrocytes after 24 h incubation. All compounds examined at 5 μg/mL and 50 μg/mL caused a statistically significant increase in caspase-3 activity. The strongest changes in the parameter studied were noted for BPAF that at a concentration of 5 μg/mL and 50 μg/mL increased caspase-3 activity up to 183% and 236.9%, respectively *versus* the control value (100%) (Fig. 3A). It was also noted that BPAF at 1 μg/mL caused a statistically insignificant increase in caspase-3 activation. Preincubation of the erythrocytes with caspase-3 inhibitor caused a significant decrease in the enzyme activity (data not shown). Fig. 3B presents a sample histogram of caspase-3 activity (FITC fluorescence) following exposure to BPS (50 μg/mL) for 24 h.

3.4. Calpain activity

After 24 incubation, BPA and its analogs activated calpain in human erythrocytes. The compounds studied at concentrations ranging from 1 to 50 μg/mL caused a statistically significant increase in the calpain activity; nevertheless at the lowest concentration (1 μg/mL) they caused small changes in the parameter under analysis. The strongest increase in calpain activation was noted in cells incubated with BPAF, which at 1 μg/mL, 5 μg/mL and 50 μg/mL increased the parameter studied up to 122.1%, 153.3% and 283%, respectively *versus* the control value (100%) (Fig. 4A). Preincubation of the erythrocytes with calpain inhibitor caused a significant decrease in the enzyme activity at 5 μg/mL and 50 μg/mL, while at 1 μg/mL the inhibitory effect was negligible (data not shown). Fig. 4B shows a sample histogram of calpain activity (CMAC fluorescence) following exposure to BPAF (5 μg/mL) for 24 h.

4. Discussion

Toxic effect of BPA on human organism has been well documented, and thus it is now partly substituted in the production of numerous products for everyday use by its chemical analogs including BPS, BPF and BPAF, whose toxicity has been poorly analyzed.

The aim of this study was to assess the effect of BPA, BPF, BPS and BPAF on eryptosis in human erythrocytes. The flow cytometry technique was used to detect changes in calcium ion levels, caspase-3 and calpain activities and phosphatidylserine (PS) translocation in this cell type.

Similarly to apoptosis of nucleated cells, eryptosis is a physiological mechanism involving elimination of damaged or aged red blood cells, and thus it is necessary for the proper functioning of the erythrocytes. It has been proven that eryptosis can be triggered by numerous stresses including xenobiotics, which can lead to excessive elimination of red blood cells from circulation, and thus may contribute to anemia and the development of other disorders [23].

For instance, Ahmed et al. [26] observed that albino adult male rats treated with BPA had reduced blood count and packed cell volume, which resulted in anemia development. Another study showed that BPA given to rats caused a decrease in the red blood cells count as well as reduced the hematocrit. The authors suggested that a decrease in the number of erythrocytes might have been due to disruption of erythropoiesis and/or increase in the destruction of red blood cells [27].

Literature data have revealed that eryptotic changes are associated with the increase in the level of intracellular calcium ions in the erythrocytes. Treatment of the erythrocytes with bisphenols, and particularly BPAF caused an increase in Ca^{2+} level (Fig. 1), the effect most likely due to activation of non-selective cation chan-

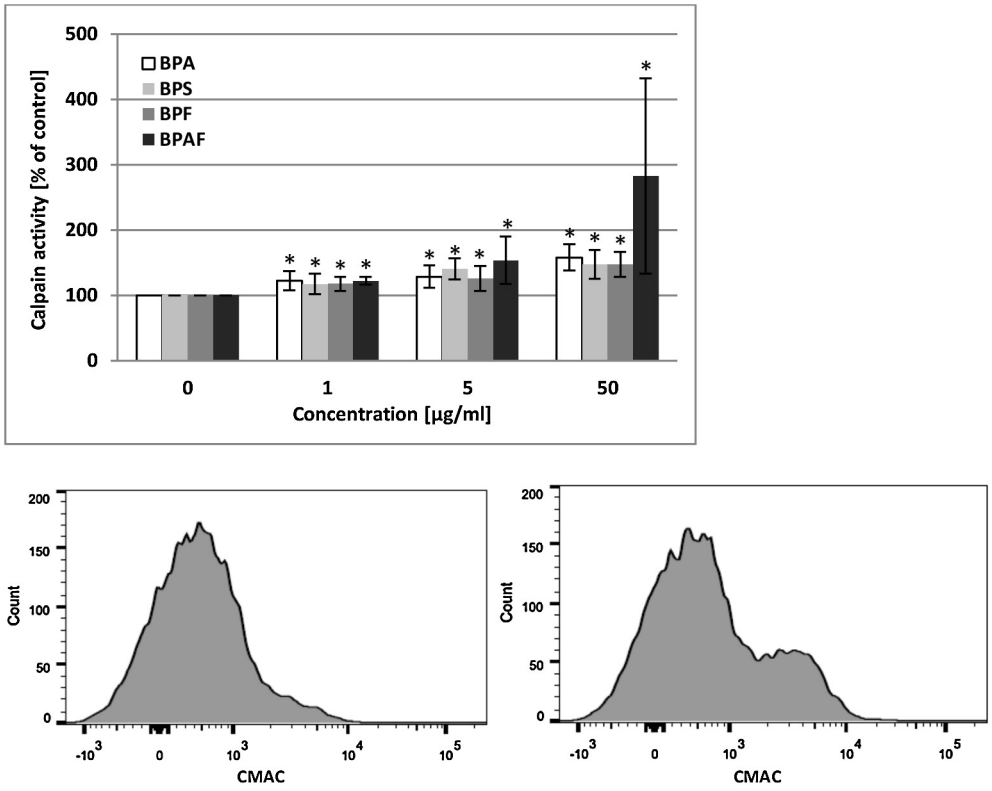

Fig. 4. Changes in calpain activity of control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 1 to 50 μg/mL for 24 h (A), $n = 6$. *Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.
Quantification of changes in CMAC fluorescence (calpain activity) in human erythrocytes exposed to BPAF. The analysis was performed by flow cytometry. Control cells, and cells treated with BPAF at 5 μg/mL (B).

nels. Although, the molecular identity of the Ca^{2+} permeable cation channels in red blood cells is still illdefined, it probably involves the transient receptor potential channel TRPC6 [28]. It has been proven that calcium ions transmit apoptotic signal in response to stress factors like xenobiotics, oxidants or prostaglandin E2 (PGE2) [23].

In previous studies, Kuo et al. [29] observed that BPA induced apoptosis in Madin–Darby canine kidney (MDCK) cells *via* an increase in cytosolic free Ca^{2+} concentrations, whereas Lee et al. [30] showed that BPAF induced apoptosis in hippocampal cell line (HT-22), which was associated with increased intracellular calcium ions level. In red blood cells, phenolic compounds has been shown to induce eryptotic changes by increasing Ca^{2+} levels. For example, Abed et al. [31] proved that polyphenol tannic acid triggered eryptosis. They also showed that suicidal erythrocyte death was significantly inhibited in the absence of Ca^{2+}.

An increase in cytosolic Ca^{2+} activity is expected to enhance lipid scrambling of the cell membrane leading to phosphatidylserine exposure at the cell surface [32]. Moreover, the elevated Ca^{2+} level activates the Ca^{2+}-dependent calpains, which proteolyse many cytoskeletal and functional proteins [33].

Calpains are intracellular cysteine proteases depending on calcium ions. Calpains may occur in red blood cells in isomeric form μ-calapin (calpain-1), which is activated by concentrations of micromolar calcium ions [34]. Activated calpains participate in eryptosis as they cleavage membrane proteins and caspases as well as inhibit anti-apoptotic proteins [23,34]. For instance, Wieschhaus et al. [35] proved that calpain-1 knockout mice showed numerous effects on erythrocyte deformability and physiology.

In this study, an increase in calpain activity was noted in red blood cells incubated with all bisphenols studied and particularly BPAF (Fig. 4). There is insufficient information concerning the effect of toxicants on calpains in living cells. Recently, Litwa et al. [36] investigated the effect of 4-para-nonylphenol on calpain activity in mouse embryonic neuronal cells; however they did not observe any activation of this enzyme. In contrast, Emerick et al. [37] noted that organophosphorous insecticide methamidophos and particularly its enantiomer increased caplain activity in hen brain and SH-SY5T neuroblastoma cells, while Chen et al. [38] showed that pesticide rotenone activated calpain in neocortical neurons undergoing apoptosis. In another study, Runge-Morris et al. [39] revealed that hydrazine and its derivatives significantly increased calpain activity in red blood cells.

Similarly to nucleated cells, caspase-3 activation in the erythrocytes is specific marker of eryptosis. The caspase-3 activation can result from xenobiotic damage or increased level of oxidative stress [40,41]. It has also been proven that active calpain can proteolytically activate caspase-3 in various cell types including erythrocytes [42].

The results of this study showed that bisphenols and BPAF in particular caused an increase in caspase-3 activity in human red blood cells (Fig. 3). In nucleated cells, Li et al. [43] demonstrated that BPA increased active caspase-3 level in Leydig cells and germ cells in mouse testis. In another study, Lee et al. [16] showed that BPA induced apoptosis in hippocampal neuronal cells by increasing intracellular calcium ions level and caspase-3 activity, while Viñas and Watson [44] noticed that BPS induced apoptosis by caspases activation in rat pituitary cell line. In the erythrocytes, phenolic compounds (flavonoids) like resveratrol, naringenin and hesperetin has been shown to activate caspase-3 [45,46].

Caspase-3 similarly to calpains is capable of degrading band 3 protein that has many structural and biochemical functions in the erythrocyte. It has been shown that clusters of degraded band 3 protein are recognized by naturally occurring antibodies, which leads

to clearance of the erythrocytes from circulation [47,48]. Moreover, caspase-3 cleavages the enzyme aminophospholipid translocase that is responsible for phospholipids translocation from internal to external leaflet of plasma membrane [49], which leads to PS accumulation and removal of red blood cells from circulation by macrophages [23,34,41].

In order to analyze phosphatidylserine translocation, we used Annexin V-FITC, which has high affinity for PS. We observed that BPA and its analogs caused a significant increase in PS translocation in human erythrocytes, while BPF and BPAF caused the strongest alterations in the parameter studied (Fig. 2).

It has been shown that phenolic compounds are capable of inducing eryptosis in human erythrocytes. Bissinger et al. [50] and Zbidah et al. [51] observed that flavones like baicalein and apigenin increased cytosolic calcium ion levels and enhanced Annexin-V-binding in human erythrocytes. Similarly, Lupescu et al. [52] noted that a pesticide rotenone raised the Ca^{2+} level and triggered PS exposure in red blood cells.

In this study, BPA and its analogs provoked eryptosis of human erythrocytes at concentrations, which may affect occupationally exposed humans. Excessive eryptosis favors development of some illnesses. It has been proven that eryptotic cells are cleared from circulation, and permanent eryptosis leads to anemia, if elimination of the erythrocytes is not fully compensated by enhanced formation of new red blood cells [53]. Moreover, Borst et al. [25] revealed that red blood cells with PS exposed on external leaflet of plasma membrane can adhere to endothelial CXCL16/SR-PSO, which may suggest that bisphenols may promote adhesion of erythrocytes to the vascular wall, and thus disturb microcirculation. The exposure of PS on erythrocyte surface is also considered to be implicated in other disorders. Recently, Ibrahim et al. [24] found a positive correlation between erythrocytes PS exposure and ferritin level in β-thalassemia patients.

Although suicidal death of erythrocytes is the key process responsible for the removal of red blood cells from circulation without inflammatory state, some studies have suggested that eryptosis may also be implicated in pathogenesis of inflammation, which may explain the effect of bisphenols on chronic diseases (type-2 diabetes, obesity, heart diseases) development [54,55]. It has been proven that various stresses including xenobiotics may increase hemoglobin's affinity for oxygen, which increase endogenous CO_2 content. Elevated carbon dioxide levels result in peroxynitrite formation, which, by displacing of zinc from RBC proteins and triggering oxidation and eryptosis, leads to releasing proinflammatory cytokines through systemic circulation, with the consequent activation of the hormonal stress response, increase in glucocorticoid levels and clustering of cardiovascular risk factors of metabolic syndrome [54–56].

5. Conclusions

(1) In this study, we showed for the first time that BPA and its analogs induced eryptosis in human erythrocytes. (2) Suicidal erythrocyte death was associated with the increase in Ca^{2+} levels, rise in calpain and caspase-3 activities and phosphatidylserine translocation. (3) Among the compounds studied, the strongest changes were induced by BPAF. (4) BPS, which is the main substituent of bisphenol A in polymers and thermal paper production exhibited similar eryptotic potential to BPA. (5) Eryptotic changes were induced by bisphenols studied at concentrations that can influence human organism as a result of occupational exposure or subacute poisoning with these substances.

Conflict of interest

None.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.12.057.

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Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (*in vitro* study)

Aneta Maćczak, Monika Cyrkler, Bożena Bukowska, Jaromir Michałowicz *

Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Łódź, Pomorska 141/143 St. 90-001 Łódź, Poland

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ABSTRACT

Bisphenol A (BPA) and its analogs are widely used in the production of various everyday use products, which leads to a common exposure of humans to these substances. The effect of bisphenols on oxidative stress parameters has not been described in detail in non-nucleated cells, therefore, we have decided to evaluate the impact of BPA and its analogs, i.e. bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF) on reactive oxygen species (ROS) formation, lipid peroxidation, glutathione (GSH) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in human erythrocytes.

The erythrocytes were incubated with the compounds studied in the concentrations ranging from 0.1 to 500 μg/ml for 1, 4 or 24 h. It has been found that bisphenols enhanced ROS (including $^{\bullet}OH$) formation, depleted GSH level, increased lipid peroxidation and changed the activities of SOD, CAT and GSH-Px. It has been noted that the strongest alterations in ROS formation, lipid peroxidation and the activity of antioxidant enzymes were induced by BPAF, which changed CAT and SOD activity even at 0.5 μg/ml. It has also been shown that BPA caused the strongest changes in GSH level, while BPS, which is the main BPA substituent in the manufacture did not alter most parameters studied.

1. Introduction

The widespread occurrence of phenols including bisphenols in aquatic and terrestrial environments and human surrounding is associated with extensive synthesis and degradation of various chemicals including epoxy resins, polycarbonates or pesticides (Michałowicz, 2014; Michałowicz and Duda, 2007; Michałowicz et al., 2008). BPA is commonly used chemical with the production estimated for above 4.5 million tons per year (Michałowicz, 2014). Moreover, from several years, an increase in the production of BPA analogs, e.g. bisphenol F (BPF), bisphenol AF (BPAF) and particularly bisphenol S (BPS) has been noted.

BPA and its analogs have been determined in tap water, bottled water, food, and particularly canned food (Michałowicz, 2014; Schecter et al., 2010; Viñas et al., 2010). Humans are also exposed to bisphenols by dust inhalation and contact of skin with thermal paper. Liao et al. (2012a, 2012b) detected BPA, BPF and BPS in dust samples collected from various microenvironments and numerous products made from thermal paper.

BPA has been detected in human organism including blood, urine and sweat (Genuis et al., 2011). BPS has also been determined in human urine (Liao et al., 2012c), while BPAF has been shown to accumulate in various tissues, urine and serum of rats (Yang et al., 2012).

BPA has been shown to provoke many adverse health effects. It exhibits estrogenic activity, and is probably carcinogenic for humans (Michałowicz, 2014; Molina et al., 2013; Vandenberg et al., 2007). The results of toxicological studies have also shown that BPA analogs reveal a similar or stronger toxic influence than BPA. Grignard et al. (2012) and Cabaton et al. (2009) showed that BPS and BPF exhibited comparable to BPA estrogenic activity, while Ruan et al. (2014) reported that BPAF revealed stronger estrogenic potential than BPA. In another studies, Wang and Zhang (2014) noted that BPS inhibited pepsin activity, while Ullah et al. (2016) showed that BPS increased reactive oxygen species (ROS) level, induced lipid peroxidation and depleted the activity of antioxidant enzymes in rat testis. In blood cells, Michałowicz et al. (2015) and Mokra et al. (2015, 2017) observed that BPA, BPS, BPF and BPAF in particular, caused necrotic, apoptotic, oxidative and genotoxic changes in human peripheral blood mononuclear cells (PBMCs), while Maćczak noticed that BPS and BPAF induced comparable or stronger than BPA eryptotic potential in human red blood cells.

The erythrocyte is a suitable research model to analyze the effect of xenobiotics on oxidative changes in living cells. Red blood cells consist of hemoglobin that binds to oxygen during its transport from the lungs to the tissues, which leads to the exposure of these cells to ROS (Bukowska et al., 2008). Red blood cells are also exposed to xenobiotics like bisphenols that enter into a human body as they participate in transport of various chemicals (Fee et al., 1975). Moreover, the erythrocytes undergo changes in the redox status of the organism, thus,

* Corresponding author.
E-mail address: michalow@biol.uni.lodz.pl (J. Michałowicz).

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alterations in this cell type are often used in diagnoses of various pathological conditions (Pandey and Rizvi, 2011).

Our previous studies have shown that phenol and its derivatives exhibited significant oxidative potential in red blood cells and caused oxidative damage in this cell type (Bukowska et al., 2009; Bukowska et al., 2008; Bukowska, 2004a). Up to now, no study has been undertaken to assess in detail the effect of BPA or its analogs on oxidative system in non-nucleated cells such as erythrocytes. That is why, the aim of this study was to evaluate the effect of BPA, BPF, BPS and BPAF on selected oxidative stress parameters such as ROS (including $^{\bullet}OH$) and reduced glutathione (GSH) levels, lipid peroxidation and the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in human red blood cells.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99%, 2,2-bis(4-hydroxyphenyl)propane)) (BPA), bisphenol F (99%, 4,4′-dihydroxydiphenylmethane) (BPF), bisphenol S (99%, 4,4′-sulfonyldiphenol) (BPS), bisphenol AF (99%, 2,2-bis(4-hydroxyphenyl)hexafluoropropane) (BPAF), 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), glutathione reductase (GR), reduced glutathione (GSH), NADPH and epinephrine were purchased from Sigma–Aldrich (USA). 3′-(p-hydroxyphenyl)-fluorescein (HPF) was bought from Molecular Probes (USA). Ethanol, chloroform, trichloroacetic acid (TCA) and other chemicals were purchased from Roth (Germany) and POCH (Poland).

Bisphenols were dissolved in ethanol (the final concentration was 0.5%). We have compared changes in the parameters studied for the samples consisting of the erythrocytes with Ringer buffer and the samples containing the erythrocytes with Ringer buffer and ethanol. As the result, we have not observed any statistically significant differences between the tested samples, which showed that ethanol concentration used in our experiments was not toxic for red blood cells.

Because BPAF has been shown to induce significant hemolysis after 1 h incubation at 500 μg/ml (nearly 30%), after 4 h incubation at 250 μg/ml (nearly 20%) and after 24 h incubation at 100 μg/ml (above 20%) (Maćczak et al., 2015), we did not perform the experiments for the above BPAF concentrations and incubation times.

2.2. Erythrocytes isolation and treatment

Leucocyte buffy-coat was taken from blood collected in Blood Bank in Łódź, Poland. Blood was collected from 20 healthy volunteers (aged 18–55) with no symptoms of infection disease. The erythrocytes were isolated according to the procedure previously described (Maćczak et al., 2015).

The cells were treated with bisphenols (final concentrations from 0.1 to 500 μg/ml) at 37 °C for 1, 4 or 24 h in the total darkness. Negative control samples consisted of the erythrocytes treated with Ringer buffer and ethanol (final concentration 0.5%).

The use of human blood (leucocyte buffy-coat) in the study of the effect of bisphenols on human erythrocytes was approved by Bioethics Committee for Scientific Investigation, University of Łódź, Poland (agreement no. KBBN-UŁ/I/7/2011).

2.3. Reactive oxygen species

The oxidation of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was analyzed by flow cytometry (Becton Dickinson, LSR II). H_2DCFDA is a stain, used for the detection of ROS (Bartosz, 2009). When H_2DCFDA diffuses across the cellular membrane, it is hydrolyzed by esterases to H_2DCF. Inside the cell, H_2DCF is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF). Positive controls consisted of hydrogen peroxide (3 mM), which was added to the erythrocytes suspension. After incubation, the cells were centrifuged (600 *g* for 10 min at 4 °C) and diluted with Ringer buffer (final density 1×10^6 cells/ml). H_2DCFDA (5 μM) was added to the samples, which were then incubated for 15 min at 37 °C in the total darkness. The analysis for 10,000 cells was performed at excitation/emission wavelengths of 488 nm and 530 nm, respectively.

2.4. Highly reactive oxygen species

Highly reactive oxygen species (mainly hydroxyl radical) were analyzed by flow cytometry (Becton Dickinson, LSR II) using 3′-(p-hydroxyphenyl)-fluorescein (HPF). HPF is nonfluorescent, until it reacts with hydroxyl radical. Oxidation of the probe results in bright green fluorescence (excitation/emission maxima – 490/515 nm). Hydroxyl radical formation (positive control) was induced by the addition of ferrous perchlorate(II) (0.2 mM) and hydrogen peroxide (2 mM) to the cells suspension. After incubation, the cells were centrifuged (600 *g* for 10 min at 4 °C) and diluted with Ringer buffer (final density 1×10^6 cells/ml). The cells were treated with HPF (4 μM) and incubated for 15 min at 37 °C in the total darkness. The data were recorded for a total of 10,000 events per sample.

2.5. Lipid peroxidation

Lipid peroxidation in human erythrocytes was analyzed by measuring of formation of thiobarbituric acid reactive substances (TBARS). The absorbance was monitored at the wavelength of 532 nm. Lipid peroxidation was expressed in absorbance units of TBARS products and showed as % of control.

2.6. Reduced glutathione

Reduced glutathione (GSH) level in the erythrocytes was determined by a modified method of Ellman (1959). The formation of 5-thio-2-nitrobenzoic acid (which was proportional to GSH concentration) was monitored at 412 nm. GSH concentration was calculated from a molar absorption coefficient for DTNB ($\varepsilon = 13.600\ M^{-1}\ cm^{-1}$) and expressed as % of the control. Mean values of controls were 1.2 and 1.16 μmol/ml packed cells after 4 h and 24 h incubation, respectively.

2.7. Antioxidat enzymes activity

Superoxide dismutase (SOD, EC.1.15.1.1) activity in the erythrocytes was determined by the method of Misra and Fridovich (1972), which is based on the ability of SOD to inhibit adrenalin self-oxidation in alkaline conditions. As a result of reaction, a colored adrenochrome is formed, while superoxide anion-radical is created as an intermediate product. SOD was determined at 480 nm, and its activity was calculated in U/mg Hb in the erythrocytes and expressed as % of the control. Mean value of control was 2041.6 U/g Hb after 24 h incubation.

The activity of glutathione peroxidase (GSH-Px, E.C.1.11.1.9) in red blood cells was assessed using tert-butyl peroxide as a substrate according to the method of Rice-Evans et al. (1991). Conversion of NADPH to $NADP^+$ was monitored at 340 nm at 25 °C for 3 min. One unit of the enzyme activity was defined as the mmol of GSH, which was converted to oxidized glutathione (GSSG/min/g Hb), using an extinction coefficient of 10^{-3} 6220 $M^{-1}\ cm^{-1}$ for NADPH (1 mmol GSH = 0.5 mmol NADPH). The final results were presented as % of the control. Mean values of controls were 30.33 and 36.73 U/g Hb after 4 h and 24 h incubation, respectively.

Catalase (CAT, EC 1.11.1.6.) activity in red blood cells was detected by the method of Aebi (1984) in which a decrease in hydrogen peroxide level was measured at 240 nm, at 25 °C for 60 s. One unit of CAT activity was defined as the enzyme activity that degraded 1 μmol of H_2O_2 in 60 s.

CAT activity was calculated in U/mg Hb in red blood cells and expressed as % of the control. Mean values of controls were 161.5 and 141.8 U/mg Hb after 4 h and 24 h incubation, respectively.

2.8. Statistical analysis

Data are shown as mean ± SD. Multiply comparisons among group mean differences were assessed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. The differences were recognized to be statistically significant when p value was lower than 0.05. Statistical analyses were conducted using STATISTICA software (StatSoft, Inc., Tulusa, USA). Mean value was counted from 4 to 6 individual experiments (donors), while for each donor, an experimental point was a mean value of 3 replications.

3. Results

3.1. ROS levels

After 1 h incubation of the erythrocytes, an increase in ROS level measured versus control sample (100%) was observed for BPA (111.3–127.9%), BPS (105.4–114.2%) and BPF (106.8–119.5%) at 250 μg/ml and 500 μg/ml, while BPAF caused an increase in this parameter (106.7–116.7%) from the concentration of 25 μg/ml (Fig. 1A). After 4 h incubation, an increase in ROS level (107.8–115.0%) was noted in cells treated with BPA (25–250 μg/ml). It was also found that BPS increased ROS level (106.9%) at 250 μg/ml, while BPF caused an increase in ROS formation (108.8–111.0%) at 100 μg/ml and 250 μg/ml. The strongest increase in ROS level (115.1–128.3%) was noted in cells treated with BPAF (5–100 μg/ml) (Fig. 1B).

A statistically significant increase in HPF oxidation (mainly $OH^{\bullet}$ formation) versus control sample (100%) was observed in red blood cells incubated with BPA (122.6%) at 500 μg/ml and BPAF (112.1–136.0%) at 100 μg/ml and 250 μg/ml for 1 h and BPAF (111.6%) at 100 μg/ml for 4 h. BPF and BPS did not change this parameter in the cells studied (data not shown).

3.2. Lipid peroxidation

After 4 h incubation, BPA (122.4–137.5%) at 100 μg/ml and 250 μg/ml, BPF (127.1%) at 250 μg/ml and BPAF (123.5–146.4%) at 25 μg/ml and 100 μg/ml caused an increase in lipid peroxidation (TBARS level) versus control sample (100%), while BPS did not induce statistically significant changes in this parameter (Fig. 2A). After 24 h incubation, an increase in lipid peroxidation was observed in red blood cells treated with BPA (126.8–154.5%) at 25 μg/ml and 100 μg/ml, BPF (131.1%) at 100 μg/ml and BPAF (111.6–146.1%) at 5 μg/ml and 25 μg/ml, while BPS did not provoke statistically significant increase in TBARS level (Fig. 2B).

3.3. GSH level

After 4 h incubation, BPA (25–500 μg/ml) decreased (92.14–68.1%) GSH level versus control sample (100%). Similarly, BPF at 250 μg/ml and 500 μg/ml depleted (87.0–80.7%) GSH content in red blood cells. Moreover, it was shown that BPAF at 100 μg/ml decreased GSH level (87.4%), while BPS did not change the parameter studied (Fig. 3A). After 24 h incubation, BPA (5–250 μg/ml) decreased GSH level (91.1–61.2%), while BPF (50–250 μg/ml) caused comparable decrease in this parameter (84.8–55.5%). It was also found that BPAF depleted GSH level (86.6%) at 25 μg/ml, while BPS did not cause statistically significant changes in this parameter (Fig. 3B).

3.4. Antioxidant enzymes activities

After 4 h incubation, a decrease in SOD activity (83.1–60.6%) was noted in cells treated with BPA at 250 μg/ml and 500 μg/ml, BPF (79.1%) at 500 μg/ml and BPAF (80.9%) at 100 μg/ml. After 24 h incubation, BPA at 100 μg/ml caused a decrease in SOD activity (82.6%), while BPF (25–100 μg/ml) more strongly decreased (85.7–72.3%) the parameter studied. The strongest decrease in SOD activity (87.4–73.0%) was noted in cells treated with BPAF that even at 0.5 μg/ml caused changes in the parameter examined. It was also noted that BPS did not provoke statistically significant alterations in the activity of SOD both after 4 h and 24 h incubation (Table 1).

After, 4 h incubation, a decrease in GSH-Px activity was noted in the erythrocytes treated with BPA (81.2–73.7%) at 250 μg/ml and 500 μg/ml and BPS at 500 μg/ml, while BPF and BPAF did not provoke statistically significant changes in the discussing parameter. After longer (24 h) incubation time, a decrease in GSH-Px activity was observed in cells incubated with BPA (84.7%) at 100 μg/ml and BPF (84.6–79.3%) at 50 μg/ml and 100 μg/ml, while BPS and BPAF did not cause statistically significant alterations in the activity of this enzyme (Table 1).

After 4 h incubation, CAT activity was depleted (82.8–64.7%) in red blood cells treated with BPA (100–500 μg/ml). Similarly, BPF at 250 μg/ml and 500 μg/ml caused a decrease (77.6–66.9%) in the activity of this enzyme. The strongest changes were noted in cells treated with BPAF, which at 25 μg/ml decreased (86.4%) the activity of CAT. After 24 h incubation, BPA (88.5–83.6%) and BPF (83.7–78.5%) in the concentrations ranging from 25 to 100 μg/ml caused a decrease in the activity of the enzyme, while BPAF even at 0.5 μg/ml and 5 μg/ml increased (117.7–112.6%) the activity of CAT. BPS did not change the parameter studied both after 4 h and 24 h incubation (Table 1).

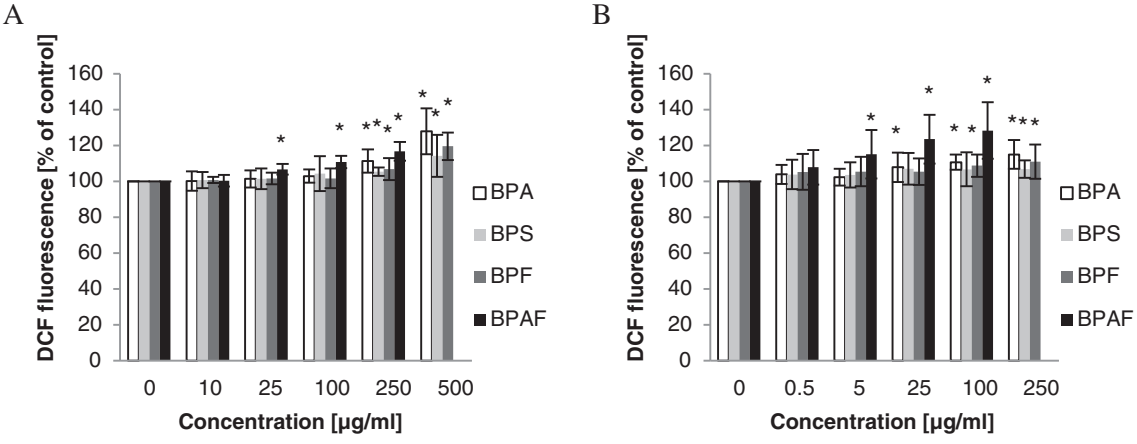

Fig. 1. Changes in ROS level in control erythrocytes and the erythrocytes incubated with BPA, BPF, BPS or BPAF in the concentrations ranging from 10 to 500 μg/ml after 1 h (A) and from 0.5 to 250 μg/ml after 4 h (B) incubation (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

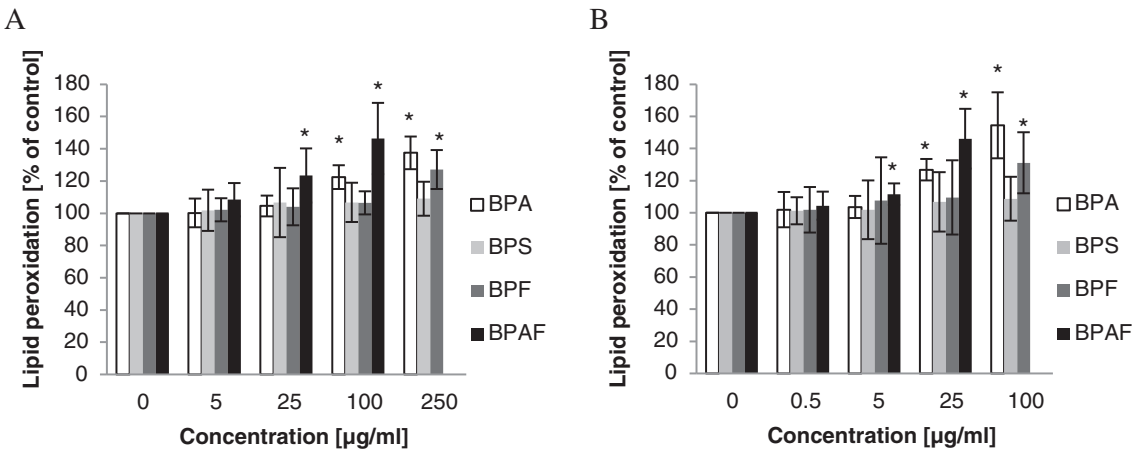

Fig. 2. Changes in lipid peroxidation level in control erythrocytes and the erythrocytes incubated with BPA, BPF, BPS or BPAF in the concentrations ranging from 5 to 250 μg/ml after 4 h (A) and from 0.5 to 100 μg/ml after 24 h incubation (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

4. Discussion

In a normal cell, a balance between the level of ROS produced during cellular metabolism and the level of antioxidants exists playing a crucial role in the defense against oxidative damage. Disruption of this balance, which may be associated both with increased ROS formation or/and decreased antioxidants levels may lead to oxidative stress (Fattman et al., 2003).

In this study, we aimed to assess the effect of BPA and its commonly used analogs, e.g. BPS, BPF and BPAF on selected oxidative stress parameters including ROS, ˙OH and GSH levels, lipid peroxidation and the activities of antioxidant enzymes, i.e. SOD, GSH-Px and CAT.

Many toxicants can exhibit adverse health effects by ROS formation, which can lead to damage to cellular structures (Bukowska et al., 2008). The researches have suggested that toxic influence of phenols is associated with ROS and/or organic radicals, e.g. semiquinones formation (Pandey and Rizvi, 2011). For instance, Atkinson and Roy (1995) showed that BPA was transformed to BPA 3,4-quinone and hydroquinone in cells playing a role in ROS formation and oxidation of cellular biomolecules.

Our results showed that bisphenols, and particularly BPAF increased H_2DCF and HPF oxidation, which proved that they induced ROS and probably other oxidants formation in human red blood cells (Fig. 1A,B).

Lee et al. (2013) observed that BPAF induced ROS formation in hippocampal cell line (HT-22), while Michałowicz et al. (2015) showed that BPA and its analogs caused an increase in ROS level in human peripheral blood mononuclear cells (PBMCs). In another studies, Xin et al. (2014) reported that BPA increased ROS formation and significantly depleted GSH level in insulinoma INS-1 cells of rats, while Ullah et al. (2016) showed that BPS increased ROS level, induced lipid peroxidation and depleted the activity of antioxidant enzymes in rat testis.

Numerous research works have also shown that an increased ROS level may lead to eryptosis of red blood cells (Lang and Lang, 2015), while our previous study proved that bisphenols, and particularly BPAF caused apoptotic alterations in human erythrocytes (Maćczak et al., 2016).

ROS and hydroxyl radical in particular, cause damage to lipid membrane, which leads to genotoxic, mutagenic and carcinogenic by-products formation (Bartosz, 2009). In this study, BPA, BPF and BPAF in particular caused a significant increase in lipid peroxidation, (Fig. 2A,B). Michałowicz et al. (2015) demonstrated that BPA and its analogs increased lipid peroxidation in PBMCs, while other researchers showed that BPA enhanced lipid peroxidation in the liver, epididymal sperm and the uterus of rats (Bindhumol et al., 2003; Chitra et al., 2003). Similarly, Leem et al. (2016) noted that BPA caused lipid peroxidation in human bone mesenchymal stem cells (hBMSC), while Abdel-Wahab (2014) reported that BPA induced lipid peroxidation and decreased the activity of antioxidant defense system in rat hepatocytes. In another study, AboulEzz and Khadrawy (2015) noticed that BPA increased malondialdehyde (MDA) level, depleted GSH level and decreased CAT and acetylcholinesterase (AChE) activities in the heart of male rats.

GSH plays a very important role in antioxidant defense system in various cell types including erythrocytes (Bartosz, 2009). Treatment of red blood cells with BPA, BPF and BPAF caused a decrease in GSH content, while BPA was shown to induce the strongest changes in this

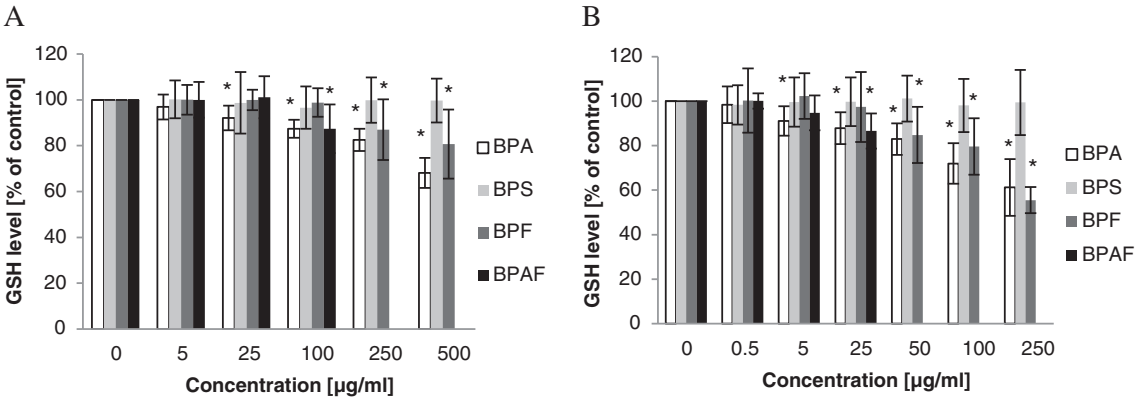

Fig. 3. The level of GSH in control erythrocytes and the erythrocytes incubated with BPA, BPF, BPS or BPAF in the concentrations ranging from 5 to 500 μg/ml after 4 h (A) and from 0.5 to 250 μg/ml after 24 h incubation (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

Table 1
Changes in the activity of antioxidant enzymes (SOD, GSH-Px, CAT) in control erythrocytes and the erythrocytes incubated with BPA, BPF, BPS or BPAF in the concentrations ranging from 10 to 500 μg/ml after 4 h (A) and from 0.1 to 100 μg/ml after 24 h incubation (B).

Compound	Concentration [μg/ml]	Activity of antioxidant enzymes [% of control]		
		SOD	GSH-Px	CAT
BPA 4 h	0	100	100	100
	10	99 ± 4	100 ± 11	97 ± 15
	25	97 ± 6	92 ± 11	93 ± 7
	100	93 ± 5	91 ± 11	83 ± 12*
	250	83 ± 10*	81 ± 13*	76 ± 14*
	500	61 ± 16*	74 ± 16*	65 ± 17*
BPA 24 h	0	100	100	100
	0.5	97 ± 13	100 ± 5	100 ± 14
	5	97 ± 12	100 ± 11	101 ± 13
	25	95 ± 13	100 ± 15	89 ± 11*
	50	94 ± 10	100 ± 16	89 ± 9*
	100	83 ± 15*	85 ± 11*	84 ± 14*
BPS 4 h	0	100	100	100
	10	96 ± 6	100 ± 20	100 ± 19
	25	95 ± 8	99 ± 16	99 ± 17
	100	98 ± 11	93 ± 13	98 ± 14
	250	96 ± 11	93 ± 15	98 ± 18
	500	98 ± 8	74 ± 20*	98 ± 16
BPS 24 h	0	100	100	100
	0.5	97 ± 6	99 ± 12	97 ± 15
	5	99 ± 13	99 ± 15	98 ± 9
	25	98 ± 14	98 ± 13	98 ± 12
	50	97 ± 13	99 ± 9	99 ± 13
	100	99 ± 13	97 ± 13	99 ± 16
BPF 4 h	0	100	100	100
	10	99 ± 9	95 ± 9	98 ± 19
	25	99 ± 10	95 ± 12	96 ± 12
	100	99 ± 8	96 ± 15	96 ± 10
	250	97 ± 10	99 ± 11	78 ± 10*
	500	79 ± 19*	98 ± 10	67 ± 13*
BPF 24 h	0	100	100	100
	0.5	100 ± 3	98 ± 14	98 ± 10
	5	97 ± 14	97 ± 15	96 ± 9
	25	86 ± 9*	99 ± 13	84 ± 8*
	50	78 ± 13*	85 ± 12*	83 ± 11*
	100	72 ± 15*	79 ± 11*	78 ± 10*
BPAF 4 h	0	100	100	100
	10	97 ± 8	97 ± 10	99 ± 10
	25	96 ± 8	98 ± 12	86 ± 9*
	100	81 ± 18*	99 ± 11	75 ± 10*
BPAF 24 h	0	100	100	100
	0.1	99 ± 11	97 ± 10	104 ± 14
	0.5	87 ± 8*	98 ± 10	118 ± 13*
	5	79 ± 12*	98 ± 15	113 ± 10*
	25	73 ± 13*	99 ± 16	103 ± 17

* Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

parameter (Fig. 3A,B). GSH level depletion may be associated with its oxidation to GSSG, protein disulfides formation or be due to conjugation of GSH with xenobiotics (Bukowska, 2004b). It has been shown that GSH level depletion is associated with Alzheimer's disease and Parkinson's disease development. Moreover, a decreased GSH content was determined in blood of diabetic patients (Bukowska, 2004a).

Antioxidant enzymes including SOD, CAT and GSH-PX remove ROS, playing an important role in a defense against oxidative stress (Pandey and Rizivi, 2011; Bartosz, 2009). SOD converts superoxide radicals to hydrogen peroxide, whereas CAT and GSH-Px catalyze the conversion of hydrogen peroxide into water (Bartosz, 2009).

BPA and its analogs changed the activity of the enzyme studied, which showed that they exhibited oxidative potential in red blood cells. Literature data has shown that BPA caused oxidative stress in rat liver by GSH level depletion and reduction of SOD, CAT, GSH-Px, glutathione-S-transferase and glutathione reductase activities (Hassan et al., 2012). Similarly, Chitra et al. (2003) reported that BPA decreased the activity of antioxidant enzymes in epididymal sperm of rats.

The superoxide radical participate in various important processes including lipid peroxidation, hemolysis and aging of red blood cells (Bartosz, 2009). It has also been shown that deficiency of SOD may lead to Heinz body formation and development of hemolytic anemia (Fee et al., 1975).

A decrease in SOD activity was noticed in the erythrocytes incubated with BPA and its analogs, particularly BPAF (Table 1). Prasanth et al. (2013) showed that BPA inhibited SOD activity isolated from human erythrocytes.

We have observed that BPA most strongly decreased the activity of GSH-Px (and depleted GSH content) (Fig. 3). GSH-Px activity is strictly associated with GSH level, which is implicated in decomposition of hydrogen peroxide. A depleted GSH level in the cell leads to a reduction of GSH-Px activity (Łukaszewicz-Hussain, 2003). Alterations in GSH-Px activity may also be associated with increased lipid peroxidation because this enzyme is capable of degrading lipid peroxides (Bukowska, 2004a).

Our study also showed that BPA, BPF and BPAF changed the activity of CAT in red blood cells (Table 1). CAT plays a crucial role in red blood cells, which are highly exposed to peroxides. A decrease in CAT or/and GSH-Px activity is associated with increased content of hydrogen peroxide in the cell (Ścibor and Czeczot, 2006). It has also been proven that an increase in CAT activity is inversely correlated with hemoglobin oxidation (Gaetani et al., 1996), and therefore, a decreased activity of this enzyme correlates with increased methemoglobin (meth-Hb) level in red blood cells. The above findings are in agreement with our earlier study, which showed that bisphenols, and particularly BPA and BPAF strongly oxidized hemoglobin in human erythrocytes (Maćczak et al., 2015). Eraslan et al. (2007) proposed two mechanisms that are responsible for a decrease in CAT activity. The first mechanism involves H_2O_2 consumption during the breakdown of free radicals and/or inhibition of CAT activity by these radicals, while the second is associated with direct inhibition of CAT activity by toxicants. It may be suggested that bisphenols studied inhibited CAT activity, and thus increased hydrogen peroxide level, which may inhibit SOD activity (Bukowska, 2004a). CAT activity depletion may be explained by –SH groups blockade at the active site of this enzyme, which leads to changes in its spacial structure (Mörikofer-Zwez et al., 1969). It is worth noticing that a reduced CAT activity accompanies many disorders such as inflammation, diabetes, atherosclerosis, and neurodegenerative diseases (Ścibor and Czeczot, 2006).

Our study showed that BPAF after longer incubation time and in low concentration caused an increase in CAT activity (Table 1). The synthesis of enzymes including CAT in the erythrocytes *in vitro* is impossible. It has been shown that hemoglobin may exhibit both catalase and peroxidase (catalase-like) activity (Giardina et al., 1995). It is probable that the oxidation of hemoglobin to met-Hb caused decomposition of hydrogen peroxide leading to the increase in CAT activity. Oxidized hemoglobin (met-Hb) reacts with H_2O_2 like peroxidase and CAT but with lower constant velocity (George and Irvine, 1952).

The results showed that BPA and its analogs were capable of inducing oxidative changes in the erythrocytes at much higher concentrations than those, which usually provoke oxidative alterations in nucleated cells. For instance, Michałowicz et al. (2015) observed that BPA, BPF, BPS and BPAF in very low concentrations (0.02–0.1 μg/ml) increased ROS level and induced lipid peroxidation in human PBMCs. Similarly, Huc et al. (2014) showed that BPA at low concentrations (10^{-8} to 10^{-9} M) increased superoxide anion level, which led to lipid peroxidation and oxidative stress in HepG2 cells. Higher resistance of erythrocytes to oxidative stress may be explained by an extensive developed enzymatic and non-enzymatic antioxidative system in this cell type. The main function of the erythrocyte is transport of oxygen, which endangers this cell to ROS, which are formed endogenously in redox reactions and autooxidation of hemoglobin. Moreover, red blood cells actively participate in neutralization of ROS that are released by exogenous systems like neutrophils, macrophages and endothelial cells present in the circulation (Mohanty et al., 2014).

It should also be mentioned that BPA, BPF and particularly BPAF caused oxidative changes in human erythrocytes at lower concentrations than those, which have been reported for phenol or its derivatives such as catechol, chlorophenols or methylphenols (Bukowska et al., 2009; Bukowska et al., 2008; Bukowska, 2004a). These findings show that bisphenols, and particularly BPA and BPAF exhibit strong oxidative potential in the erythrocytes, which are considered (as described above) to be highly resistant to oxidative stress and damage induced by various xenobiotics.

Oxidative stress has been shown to be involved in aging and various diseases development. Hybertson et al. (2011) reported that oxidative stress may contribute to the development and progression of diabetes, cardiovascular disorders, rheumatoid arthritis, pneumonia, hepatitis, neurological diseases, cataract, glaucoma and cancer.

Some reports have also shown that BPA may cause development of some diseases through induction of oxidative stress. For instance, Moghaddam et al. (2015) suggested that BPA may cause hyperglycemia in adult male mice through oxidative stress induction, while Eid et al. (2015) reported that in liver of female rats BPA increased oxidative/nitrosative stress, decreased antioxidant enzyme activities and caused chronic inflammation. Similarly, Yang et al. (2009) showed that urinary BPA levels were positively associated with the concentrations of 8-hydroxydeoxyguanosine (8-OHdG), C-reactive protein (CRP) and MDA in blood of postmenopausal women, suggesting that exposure to BPA may cause oxidative stress and inflammation.

5. Conclusions

(1) Our study for the first time has illustrated the mechanism of oxidative action of BPA and its analogs, i.e. BPS, BPF and BPAF in non-nucleated cells such as mature erythrocytes. (2) The compounds studied increased ROS (including $^{\bullet}OH$) formation, decreased GSH level, caused lipid peroxidation and altered the activities of SOD, CAT and GSH-Px. (3) Comparison of the action of BPA and its analogs showed that BPAF exhibited the strongest oxidative potential in red blood cells. (4) BPS, which is the main substituent of bisphenol A in polymers and thermal paper production exhibited much lower oxidative potential than BPA. (5) Changes in the parameters studied occurred at bisphenols concentrations that may affect human organism during occupational exposure (BPAF, BPA) (He et al., 2009) or acute poisoning with these substances.

The authors declare that there is no conflict of interest.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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The *in vitro* comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocyte membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na^+/K^+ ATPase and AChE activities

Aneta Maćczak, Piotr Duchnowicz, Paulina Sicińska, Maria Koter-Michalak, Bożena Bukowska, Jaromir Michałowicz*

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ABSTRACT

Bisphenols are massively used in the industry, and thus the exposure of biota including humans to these substances has been noted. In this study we have assessed the effect of BPA and its selected analogs, i.e. BPS, BPF and BPAF on membrane of human red blood cells, which is the first barrier that must be overcome by xenobiotics penetrating the cell, and is commonly utilized as a model in the investigation of the effect of different xenobiotics on various cell types. Red blood cells were incubated with BPA and its analogs in the concentrations ranging from 0.1 to 250 μg/ml for 4 h and 24 h. We have noted that the compounds studied altered membrane fluidity at its hydrophobic region, increased internal viscosity and osmotic fragility of the erythrocytes and altered conformational state of membrane proteins. Moreover, bisphenols examined increased thiol groups level, caused oxidative damage to membrane proteins, decreased ATP level, depleted the activity of Na+/K + ATPase and changed the activity of AChE in human red blood cells. It has been shown that the strongest changes were noted in cells treated with BPAF, while BPS caused the weakest (or none) alterations in the parameters studied.

1. Introduction

Phenols including bisphenols are one of the most widespread group of chemicals in the environment and human surrounding (Michałowicz et al., 2008; Igbinosa et al., 2013). Bisphenol A (BPA) is a chemical substance used in large amounts in the production of polycarbonate polymers (plastic food containers, bottles, toys, medical equipment), epoxy resins (cans, water pipes) and thermal paper (receipts, tickets, newspapers) (Michałowicz, 2014). A worldwide production of BPA in 2013 was estimated for 6,8 million tons (GrandViewResearch, 2014).

The exposure of the general population to BPA and its analogs occurs mainly through food and, to a lesser extent, through drinking water (Michałowicz, 2014). Another routes of human exposure to BPA are inhalation of dust and dermal contact with thermal paper. Bisphenols including BPA, BPS and BPF have been determined in the air microenvironments, home dust and in the products made from thermal paper e.g receipts or bills (Vandenberg et al., 2007; Liao et al., 2012a, 2012b).

BPA, BPS, BPF and BPAF have been determined in tissues including blood and adipose tissue and in the urine of humans environmentally and occupationally exposed (Calafat et al., 2008; He et al., 2009; Genuis et al., 2011; Liao et al., 2012b; Vandenberg et al., 2012; Rocha et al., 2016).

Numerous studies have shown that BPA has adverse health effects. BPA has been shown to exhibit endocrine disrupting activity and it is supposed to be cancerogenic for humans (Vandenberg et al., 2009; Rutkowska and Rachon, 2014; Fic et al., 2015). Moreover, epidemiological surveys have revealed association between an increased BPA level in blood or urine of the general population and development of diabetes, obesity, asthma or heart disease (Cabaton et al., 2011; Shankar et al., 2012; Sengupta et al., 2013; Michałowicz, 2014).

For several years, an increase in the production of other bisphenols including bisphenol F (BPF), bisphenol AF (BPAF) and particularly bisphenol S (BPS) has been noted (Lotti et al., 2013), which is the result of ban on BPA in the products for infants as well as packagings and bottles in some countries (USA, Canada, European Union) (Liao and Kannan, 2013). Several research works have proven that BPA analogs may demonstrate a similar or even a stronger toxic influence than BPA itself. For instance, in blood cells including erythrocytes, BPS and BPF have been shown to exert comparable, whereas BPAF even stronger toxic

* Corresponding author. Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Łódź, Pomorska 141/143 St. 90-001, Łódź, Poland.
E-mail address: jaromir.michalowicz@biol.uni.lodz.pl (J. Michałowicz).

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potential than BPA (Maćczak et al., 2015, 2016; 2017; Michałowicz et al., 2015; Mokra et al., 2015, 2017).

Cellular membrane is the first barrier that must be overcome by xenobiotics passing into the cell, therefore it is highly exposed to their action. Damage to cellular membrane may alter cell function, and ultimately cause cell death (Duchnowicz and Koter, 2002). Changes in cell membrane properties may also be implicated in development of various diseases including anemia, diabetes, heart disease and cancer (Baritaki et al., 2006; Pytel et al., 2013; Kroger et al., 2015).

Some studies have shown that phenolic compounds may alter erythrocyte membrane properties (Bukowska et al., 2009). Moreover, our previous study revealed that BPA and its analogs caused morphological changes in human red blood cells (Maćczak et al., 2015), which was probably associated with alteration or/and damage of membrane components of this cell type. Recently, we have noticed that BPA and it analogs induced oxidative stress and caused lipid peroxidation in red blood cells, which could have contributed to deterioration of the structure of erythrocyte membrane (Maćczak et al., 2017).

In this study, we have decided to look closer into the mechanism of action of BPA, BPS, BPF and BPAF on membrane of red blood cells. We have assessed changes in osmotic fragility and internal viscosity of erythrocytes as well as perturbations in membrane fluidity and conformational state of membrane proteins in this cell type. Moreover, we have evaluated changes in sulfhydryl groups level, protein peroxidation, ATP level as well as alterations in Na^+/K^+ ATPase and acetylcholinesterase (AChE) activity in human red blood cells.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99%, 2,2-bis(4-hydroxyphenyl)propane) (BPA), bisphenol F (99%, 4,4′-dihydroxydiphenylmethane) (BPF), bisphenol S (99%, 4,4′-sulfonyldiphenol) (BPS), bisphenol AF (99%, 2,2-bis(4-hydroxyphenyl)hexafluoropropane) (BPAF), 5-doxylstearic acid (5-DSA), 16-doxylstearic acid (16-DSA), 4-N-maleimide-2,2,6,6-tetramethylopiperidine-1-oxyl (MSL), 2,2,6,6-tetramethyl piperidine-N-oxyl-4-amine (TEMPAMINE), ouabain, acetylthiocholine iodide were purchased from Sigma-Aldrich, USA. ATP Determination Kit was bought in Molecular Probes, USA. EDTA, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and other chemicals were obtained from Roth, Germany and POCh, Poland.

2.2. Erythrocyte isolation and treatment

Human erythrocytes were isolated from leucocyte buffy-coat obtained from 25 healthy donors (aged 18–57) with no symptoms of infection disease. The blood was collected in the Blood Bank in Lodz, Poland. Red blood cells isolation was performed according to the procedure described previously by Maćczak et al. (2015). The erythrocytes at 5% hematocrit were suspended in Ringer buffer and incubated at 37 °C with BPA or its analogs (dissolved in ethanol - final concentration in the sample was 0.5%). The samples that contained the erythrocytes and Ringer buffer and ethanol (0.5%) were used as negative controls.

Bisphenols were dissolved in ethanol (the final concentration of ethanol in the samples was 0.5%). We have compared changes in all parameters studied between the samples containing the erythrocytes with Ringer buffer and the samples consisting of the erythrocytes with Ringer buffer and ethanol. Because we have not observed any statistically significant differences between the samples studied, we considered that ethanol concentration used in the experiments was not toxic for red blood cells.

The erythrocytes were treated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.5 to 250 μg/mL and from 0.1 to 100 μg/mL and incubated for 4 h and 24 h, respectively. The incubation was conducted in the total darkness.

Because BPAF has been shown to induce significant hemolysis after 4 h incubation at 250 μg/ml (nearly 20%) and after 24 h incubation at 100 μg/ml (above 20%) (Maćczak et al., 2015), we have not performed the experiments for the above BPAF concentrations and incubation times.

2.3. Isolation of erythrocyte membranes

The erythrocyte membranes were prepared by the method of Dodge et al. (1963) with modification. After the incubation, the erythrocytes were centrifuged at 3000 rpm for 5 min at 4 °C. Then, the cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) supplemented with 1 mM EDTA and 0.01% PMSF on ice. After 10 min, the erythrocytes were centrifuged at 14000 rpm for 5 min at 4 °C. The samples were washed several times with the above-mentioned buffer, until white, purified erythrocyte membranes were obtained. The buffer was cooled to 4 °C and the whole preparation procedure was conducted in the ice-bath conditions.

The protein content in membrane preparation was determined according to the method of Lowry et al. (1951). The absorbance was measured at a wavelength of 715 nm. The concentration of protein in the sample was read from a calibration curve in the concentrations range from 30 to 300 μg proteins/ml using bovine serum albumin as the standard.

The erythrocyte membranes were used for determination W/S ratio, thiol groups level, protein oxidation and Na^+/K^+ ATPase activity.

2.4. Membrane fluidity

The fluidity of erythrocyte membrane was analyzed by electron paramagnetic resonance (EPR) spectroscopy (Brucker 300 Spectrometer) using two spin labeled fatty acids, which reside at different depths within the lipid bilayer: 5-doxylstearic acid (5-DSA) and 16-doxylstearic acid (16-DSA). The order parameter S (determined by the use of 5-DSA) is a degree of the distribution of molecular orientations with respect to a reference axis, chosen in this study to be normal to the membrane surface. An increase in S order parameter reflects a decrease in the segmental flexibility of the spin sample. Order parameter S was calculated as described by Koter et al. (2004). The relaxation times of τ_B and τ_C were determined using 16-DSA. The correlation time τ_B reflects the motion in the direction perpendicular, and τ_C describes the motion in the direction parallel to the long axis of a lipid molecule. The erythrocytes were labeled with the spin labels, incubated for 30 min at room temperature, and then analyzed using EPR. Changes in S order parameters were expressed as % of control Changes in τ_B and τ_C parameters were calculated in s and expressed as % of control. Mean values of controls of τ_B parameter were $1{,}83 \times 10^{-8}$ s and $1{,}86 \times 10^{-8}$ s after 4 h and 24 h incubation, respectively, while mean values of controls of τ_c parameter were $2{,}49 \times 10^{-8}$ s and $2{,}40 \times 10^{-8}$ s after 4 h and 24 h incubation, respectively.

2.5. W/S ratio

Parameter W/S that defines the conformation state of internal proteins was determined using a spin label MSL, which covalently binds proteins. A protein molecule usually contains several thiol groups differently available for the label molecule, thus in different way limits its rotation. High and low-field lines of EPR spectrum were divided into peaks resulting from weakly (W) and strongly (S) immobilized components. Changes in the W/S ratio reflect the conformational alterations of the labeled molecule during protein conformational movements. A volume of 1.5 μL of 20 mM of MSL solution was added to 150 μL of membrane suspension (0.2 mM of MSL), and incubated for 12 h at 4 °C. After incubation, the excess of the label was washed out and ESR spectrum (Brucker 300 Spectrometer) was performed. Then, the W/S ratio was calculated and showed as % of control.

2.6. Osmotic fragility

Colorimetric method was employed to measure the osmotic fragility of red blood cells. After incubation of the erythrocytes with BPA or its analogs, the cells were centrifuged at 1200 rpm for 5 min at 4 °C. Then, the cells were washed with 0.9% NaCl; this step was repeated three times. After removing the supernatant of the last centrifugation, red blood cells were suspended in NaCl solution in the concentrations range from 0.1 to 0.9% (13 points were selected). The cells were incubated for 60 min at 37 °C, and then centrifuged at 3000 rpm for 10 min at 4 °C. Optical density of the supernatant was measured at 540 nm using microplate absorbance reader (BioTek Elx808, BIOTEK). Percent of hemolysis was calculated according to the procedure described previously by Maćczak et al. (2015).

2.7. Internal viscosity

The rotational dynamics of TEMPAMINE was used to study directly the intracellular environment. The extracellular signal from TEMPAMINE was broadened away by the use of potassium ferricyanide, which did not enter the cell. In the study 1 mM TEMPAMINE, 120 mM ferricyanide, and the erythrocytes were mixed together to detect EPR signal that arose from the label only in the intracellular aqueous space of red blood cells. The analysis was conducted using Brucker 300 Spectrometer. The changes in the parameter studied were calculated in Pa x s and expressed as % of control. Mean value of control after 4 h incubation was 0.249 Pa x s.

2.8. Thiol groups level

The concentration of thiol (-SH) groups in the membranes was performed by the method of Ellman (1959). The erythrocyte membranes were diluted in 10% SDS, and then incubated in the presence of 0.3 M Na_2HPO_4 and 4 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 1% sodium citrate at room temperature. After 30 min incubation, the absorbance was measured at a wavelength of 412 nm. The level of -SH groups was calculated using a milimolar absorption coefficient for DTNB. The results were expressed as -SH nmol/mg proteins and showed as % of control. Mean values of controls were 71.7 nmol and 72.6 nmol -SH/mg protein after 4 h and 24 h incubation, respectively. Duchnowicz et al. (2005) determined comparable –SH groups level (mean 60 nmol/mg protein) in control samples (membranes) of human erythrocytes.

2.9. Protein damage

Analysis of protein damage was based on the measurement of sample fluorescence at 335 nm after excitation at 295 nm. Fluorescent properties of proteins are associated with the presence of aromatic aminoacids (mainly tryptophan) in their structure. A decrease in fluorescence of the sample results from oxidative damage to tryptophan, and thus damage to proteins of the erythrocyte membrane (Vivian and Callis, 2001). After the incubation, the cells were centrifuged (14000 rpm for 10 min at 4 °C) and the erythrocytes membranes were isolated. The analysis was performed in 96-well plates using a microplate reader (Cary Eclipse, Varian). The results were presented as % of control.

2.10. Na^+/K^+ ATPase activity

ATPase activity was measured according to the method described by Bartosz et al. (1994). This method is based on the measurement of orthophosphate released from ATP during the incubation of erythrocyte membranes with a medium (10 mM $MgCl_2$, 85 mM NaCl, 10 mM KCl, 100 mM Tris-HCl buffer, 1 mM ATP, pH 7.4) with and without 0.1 mM ouabain, which is added to inhibit the Na^+/K^+ ATPase activity. The membranes were incubated in incubation medium with and without ouabain for 30 min at 37 °C and 0 °C. After that, 0.6 M TCA was added to the samples, which were incubated for 3 min at room temperature, and then centrifuged (14000 rpm for 5 min at 4 °C). Subsequently, the concentration of released orthophosphate was determined by the method of Van Veldhoven and Mannaerts (1987) with malachite green in a solution of polyvinyl alcohol, based on the calibration curve using KH_2PO_4 as the standard. The absorbance was performed at 610 nm for membranes incubated in the absence (total ATPase activity) and the presence (Na^+/K^+ ATPase activity) of ouabain (BioTek Elx 808, BIOTEK). Na^+/K^+ ATPase activity was calculated as the difference between the activity of ATPase without and with ouabain in incubation medium. The results were expressed in nmol orthophosphate/mg proteins × h and presented as % of control. Mean values of controls were 981.9 nmol and 702.6 nmol *o*-phosphate/mg protein x h after 4 h and 24 h incubation, respectively. The obtained values were higher than those for Na^+/K^+ ATPase activity (485.3 nmol *o*-phosphate/mg protein x h) determined in erythrocyte membrane of healthy subjects as reported by Bukowska et al. (2015).

2.11. ATP level

Intracellular ATP level in the erythrocytes was determined on the basis of oxidative decarboxylation of luciferin by firefly luciferase in the presence of ATP and magnesium ions with production of bioluminescence. The amount of light emission was linearly related to the intracellular ATP concentration (Stanly and Williams, 1969). The level of ATP in the erythrocytes was measured using ATP Determination Kit (Molecular Probes, USA). After the incubation, the samples were centrifuged (14000 rpm for 10 min at 4 °C), and then cellular ATP was extracted using 1 ml boiling deionized water, which was added to the cell pellet according to the method of Yang et al. (2002). The cells were vortexed and centrifuged (14000 rpm for 10 min at 4 °C). The supernatant (cell lysate) was collected and placed in 96-well plates. Subsequently, a volume of 100 µl of luciferin/luciferase mixture was added to 20 µl of the lysed cell suspension and incubated for 20 min at room temperature in the total darkness. The light emission was measured at 590 nm (Fluoroskan Ascent FL, Thermo Scientific). The concentration of ATP was calculated in nM packed cells and shown as % of control. Mean values of controls were 1.38 nM and 1.34 nM of ATP/packed cells, after 4 h and 24 h incubation, respectively. Agalakova and Gusev (2012) detected similar ATP content (mean 1.26 nM packed cells) in control erythrocytes.

2.12. Acetylcholinesterase activity

The activity of acetylcholinesterase (AChE) was assayed by the method of Ellman et al. (1961) in which acetylthiocholine iodide is hydrolyzed by AChE to acetic acid and thiocholine. The erythrocytes were diluted with 5 mM phosphoric buffer (pH 8.0) to 0.05% hematocrit. To a volume of 2 mL of the erythrocyte suspension 20 µl of 10 mM DTNB in 18 mM $NaHCO_3$ was added. Immediately before measurement, to the cuvette containing 2 mL of the sample, 60 µl of acetylthiocholine iodide at a concentration of 10 mM was added. The absorbance slope was measured for 1 min at the wavelength of 412 nm (Specord 250 Plus, Analytik Jena AG). One unit of AChE activity was defined as the amount of µmol of acetylthiocholine degraded in 1 min by AChE contained in 1 mL of the erythrocytes at 100% hematocrit (packed cells). The final results were expressed as % of control. Mean values of controls were 7.50 µmol and 7.17 µmol/min/ml packed cells after 4 h and 24 h incubation, respectively. The above values are comparable to the control values of AChE activity (mean 9.37 µmol/min/ml packed cells) determined in human erythrocytes in the study of Kwiatkowska et al. (2014).

Table 1
Changes in parameter S and correlation times of τ_B and τ_C in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 5 to 250 μg/ml for 4 h and from 0.5 to 100 μg/ml for 24 h (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

Compound	Concentration [μg/ml]	Order parameter S [%]	Correlation times τ_B [%]	Correlation times τ_C [%]
BPA 4 h	0	100 ± 0	100 ± 0	100 ± 0
	5	100 ± 2.0	96 ± 8.8	95 ± 6.6
	25	100 ± 1.5	92 ± 4.8	92 ± 4.3
	100	101 ± 1.0	92 ± 4.5	91 ± 4.7*
	250	100 ± 1.7	89 ± 6.5*	85 ± 8.6*
BPA 24 h	0	100 ± 0	100 ± 0	100 ± 0
	0.5	100 ± 0.6	101 ± 4.7	104 ± 4.1
	5	100 ± 0.8	92 ± 5.3*	91 ± 4.7*
	25	101 ± 0.6	92 ± 5.0*	88 ± 6.8*
	100	102 ± 1.1	88 ± 6.2*	87 ± 7.2*
BPS 4 h	0	100 ± 0	100 ± 0	100 ± 0
	5	99 ± 1.4	101 ± 6.1	101 ± 3.3
	25	99 ± 1.0	101 ± 10.2	101 ± 8.2
	100	99 ± 0.8	101 ± 6.4	100 ± 7.1
	250	99 ± 1.5	97 ± 8.6	96 ± 6.9
BPS 24 h	0	100 ± 0	100 ± 0	100 ± 0
	0.5	100 ± 1.1	100 ± 7.8	99 ± 2.8
	5	100 ± 0.5	98 ± 9.1	97 ± 6.9
	25	100 ± 2.5	98 ± 3.6	96 ± 3.8
	100	101 ± 1.7	95 ± 6.4	96 ± 4.7
BPF 4 h	0	100 ± 0	100 ± 0	100 ± 0
	5	100 ± 1.4	97 ± 4.4	97 ± 6.2
	25	100 ± 1.3	98 ± 7.6	99 ± 2.1
	100	101 ± 1.6	95 ± 5.2	90 ± 4.7*
	250	103 ± 1.5	96 ± 5.2	91 ± 5.5*
BPF 24 h	0	100 ± 0	100 ± 0	100 ± 0
	0.5	100 ± 1.1	98 ± 4.1	98 ± 3.4
	5	99 ± 0.4	93 ± 4.9*	92 ± 3.3*
	25	100 ± 0.8	92 ± 3.8*	91 ± 4.7*
	100	101 ± 0.7	87 ± 4.5*	88 ± 4.1*
BPAF 4 h	0	100 ± 0	100 ± 0	100 ± 0
	5	100 ± 1.1	97 ± 4.9	100 ± 5.6
	25	102 ± 1.6	90 ± 3.5*	101 ± 5.0
	100	102 ± 1.5	103 ± 4.5	106 ± 4.4
BPAF 24 h	0	100 ± 0	100 ± 0	100 ± 0
	0.5	100 ± 0.8	93 ± 2.9*	92 ± 6.1*
	5	101 ± 0.8	91 ± 3.1*	88 ± 7.0*
	25	102 ± 1.0	87 ± 11.8*	99 ± 8.7

2.13. Statistical analysis

The results were presented as mean ± SD achieved from 4 to 6 individual experiments (4–6 blood donors), whereas for each individual (donor), an experimental point was a mean value of 2–3 replications. Multiply comparisons among group mean differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. The differences were considered to be statistically significant for $P < 0.05$. All statistical analyses were performed using STATISTICA software (StatSoft, Inc, Tulusa, USA).

3. Results

3.1. Membrane fluidity

The study showed that bisphenols studied did not provoke statistically significant alterations in the value of parameter S in human erythrocytes both after 4 h and 24 h incubation (Table 1). Differently, it was observed that BPA and its analogs caused changes in τ_B and τ_C correlation times. After 4 h incubation, BPA at 250 μg/ml and BPAF at 25 μg/ml caused statistically significant decrease of τ_B correlation time. A decrease in this parameter was also noted after 24 h incubation of the cells with BPA and BPF in the concentrations ranging from 5 to 100 μg/ml and with BPAF in the concentrations range from 0.5 μg/ml to 25 μg/ml (Table 1). After 4 h incubation, a statistically significant decrease of correlation times τ_C was induced by BPA and BPF at 100 μg/ml and 250 μg/ml, while BPAF decreased this parameter at 25 μg/ml. After 24 h incubation, BPA and BPF in the concentrations ranging from 5 to 100 μg/ml and BPAF at 0.5 μg/ml and 5 μg/ml caused a decrease in correlation time τ_C. The strongest changes in τ_B and τ_C correlation times were noted in cells treated with BPAF. It was also noticed that BPS did not cause statistically significant alterations in these parameters both after 4 h and 24 h incubation (Table 1).

3.2. W/S ratio

All bisphenols studied caused an increase in W/S ratio in human erythrocytes. After 4 h incubation, BPA, BPS, BPF and BPAF in the concentrations ranging from 0.5 to 100 μg/ml caused statistically significant increase in the parameter studied (Fig. 1A). After 24 h incubation, an increase in W/S ratio was observed in cells incubated with BPA, BPF and BPAF in the concentrations ranging from 0.1 to 25 μg/ml, while BPS increased this parameter in the concentrations ranging from 0.5 to 25 μg/ml (Fig. 1B). The highest increase in W/S ratio was noted for BPAF, while the lowest for BPS (Fig. 1A and B).

3.3. Internal viscosity

It was observed that after 4 h incubation BPA (108 ± 4%) and BPF (104.8 ± 6%) at 250 μg/ml and BPAF at 100 μg/ml (129.7 ± 7%) caused an increase in internal viscosity of human erythrocytes, while

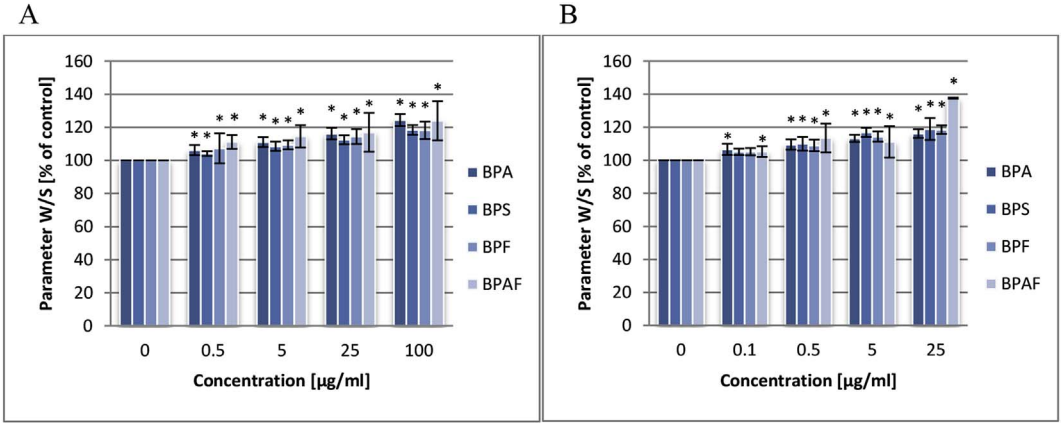

Fig. 1. Changes in W/S parameter in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.5 to 100 μg/ml for 4 h (A) and from 0.1 to 25 μg/ml for 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

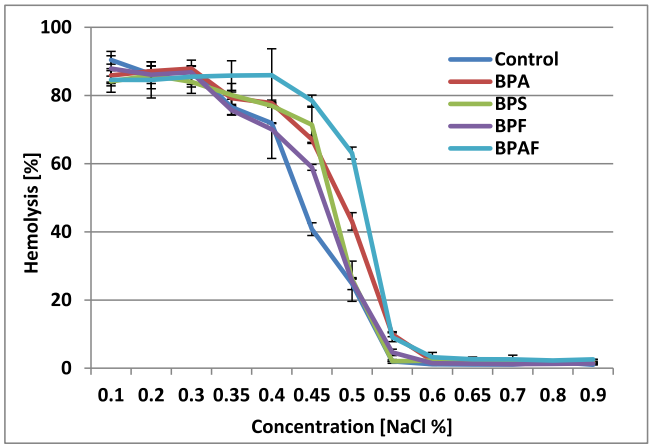

Fig. 2. Changes in osmotic fragility of human control erythrocytes and erythrocytes treated with BPA, BPS, BPF or BPAF at 100 μg/ml for 4 h, and then incubated with NaCl in the concentrations range from 0.1 to 0.9% for 1 h.

BPS did not change the parameter studied (99.9 ± 2%) versus control sample (100%) (data not shown).

3.4. Osmotic fragility

We have noticed that bisphenols studied at 100 μg/mL (after 4 h incubation) increased osmotic fragility of human red blood cells. The strongest changes were observed in cells treated with BPA, and particularly BPAF (Fig. 2).

3.5. Thiol groups level

After 4 h incubation, BPA and BPAF in the concentrations ranging from 5 to 100 μg/ml caused an increase in thiol groups level in human erythrocytes (Fig. 3A). After 24 h incubation BPS at 5 μg/ml and 25 μg/ml and BPAF in the concentrations ranging from 0.5 to 25 μg/ml increased -SH group level in red blood cells (Fig. 3B).

3.6. Protein damage

It was observed that BPA and its analogs decreased tryptophan fluorescence, which showed that these substances induced oxidative damage to protein in human erythrocytes. After 4 h incubation, BPA, BPF and BPS in the concentrations ranging from 5 to 250 μg/ml decreased tryptophan fluorescence, while BPAF reduced this parameter in the concentrations ranging from 5 to 100 μg/ml (Fig. 4A). After 24 h incubation BPA and all of its analogs in the concentrations ranging from 0.5 to 100 μg/ml caused a decrease in tryptophan fluorescence but the effects were more intense (Fig. 4B). The results showed that BPAF caused the strongest oxidative damage to proteins, while BPS induced the weakest changes in the examined parameter (Fig. 4A and B).

3.7. Na^+/K^+ ATPase activity

The compounds studied decreased the activity of Na^+/K^+ ATPase in red blood cells. After 4 h incubation, a decrease of ATPase activity was noted in cells incubated with all compounds studied in the concentrations ranging from 5 to 100 μg/ml, while the strongest changes were noted for BPA and BPS, and particularly for BPAF (Fig. 5A). A statistically significant decrease in Na^+/K^+ ATPase activity was also observed in red blood cells incubated for 24 h with BPA and its analogs in the concentrations ranging from 0.5 to 25 μg/ml (Fig. 4B).

3.8. ATP level

It was found that BPA and its analogs (excluding BPS) reduced intracellular ATP level in human erythrocytes. BPA at 25 μg/ml and BPAF in the concentrations ranging from 0.5 to 25 μg/ml caused statistically significant decrease in ATP level after 4 h incubation, while BPS and BPF did not cause statistically significant alterations in this parameter (Fig. 6A). After 24 h incubation, BPA at 5 μg/ml and 25 μg/ml caused statistically significant decrease in ATP level, while BPF at 25 μg/ml depleted the parameter studied. The strongest changes were noted for BPAF, which in the concentrations ranging from 0.1 to 25 μg/ml strongly decreased ATP content (Fig. 6B).

3.9. Acetylcholinesterase activity

The study showed that all bisphenols studied changed the activity of AChE in red blood cells. After 4 h incubation, BPA and BPAF at 100 μg/ml caused statistically significant increase and decrease of AChE activity, respectively, whereas BPS and BPF did not cause changes in this parameter (Fig. 7A). After 24 h of incubation, BPA, BPS, BPF and BPAF at 5 μg/ml and 25 μg/ml caused statistically significant increase in the AChE activity (Fig. 7B). It was noted that BPA and BPAF in particular more strongly altered AChE activity.

4. Discussion

In this study, the effect of BPA and its selected analogs, i.e. BPS, BPF and BPAF on erythrocyte membrane properties was studied, which is a model in the investigation of the effects of different xenobiotics on various cell types.

Changes in cell membrane properties including alterations in its fluidity may influence cell functions including changes in the enzymes activity (Tang et al., 2008) and may be implicated in development of

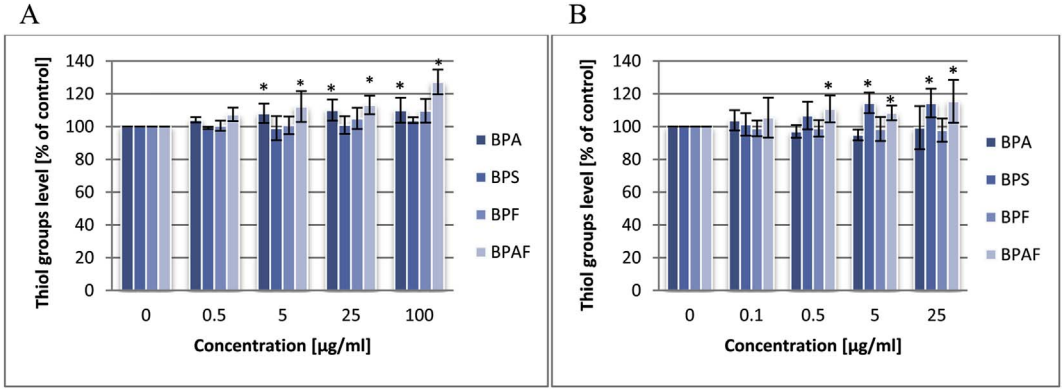

Fig. 3. Changes in the thiol groups level in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.5 to 100 μg/ml for 4 h (A) and from 0.1 to 25 μg/ml for 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

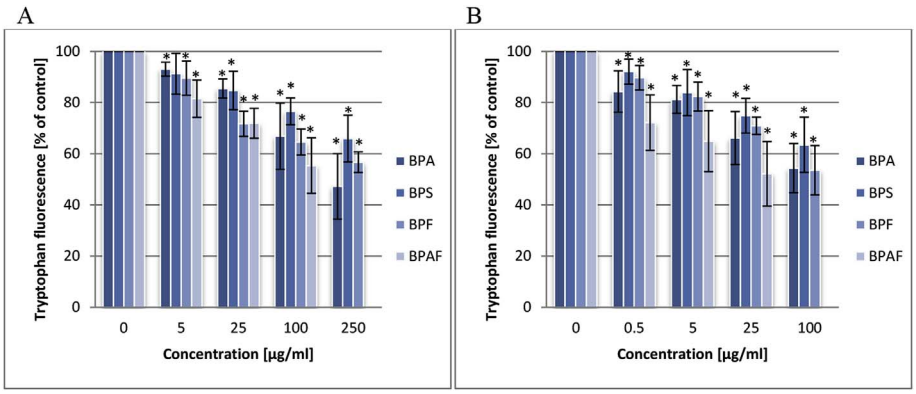

Fig. 4. Changes in tryptophan fluorescence (protein damage) in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 5 to 250 µg/ml for 4 h (A) and from 0.5 to 100 µg/ml for 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

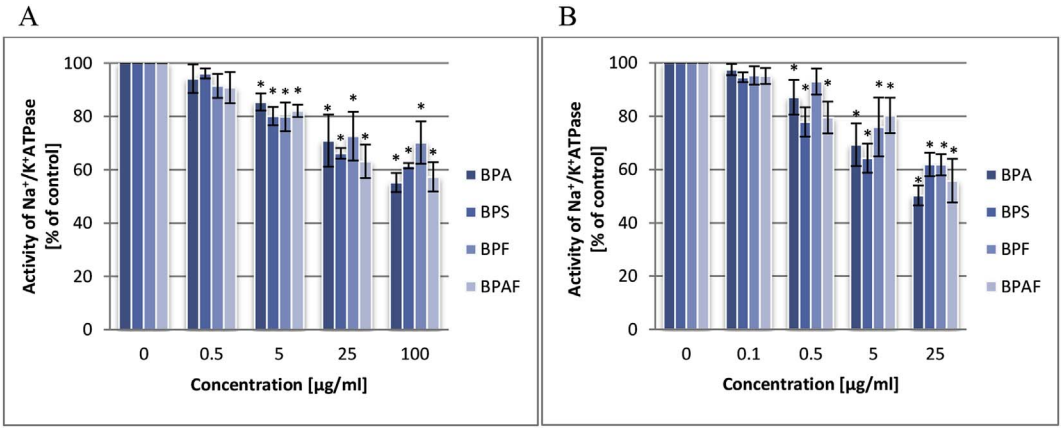

Fig. 5. Changes in Na+/K + ATPase activity in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.5 to 100 µg/ml for 4 h (A) and from 0.1 to 25 µg/ml for 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

diabetes, heart disease and cancer (Baritaki et al., 2006; Pytel et al., 2013; Kroger et al., 2015).

In the present study, the erythrocytes were labeled with 5-DSA to determine order parameter S, which describes changes in fluidity of hydrophilic region of cellular membrane. The results showed that BPA and its analogs did not change the value of parameter S, which showed that they were not located at the level of 5th carbon atom of the lipid membrane of human red blood cells (Table 1). Differently, the compounds examined disturbed hydrophobic regions of erythrocytes membrane labeled with 16-DSA (for this analysis, relaxation times of τ_B and τ_C were determined). The changes were noticed in red blood cells incubated with BPA, BPF and particularly with BPAF (Table 1). The most probably, BPA and its analogs due to significant hydrophobicity (log $K_{o/w}$ value for BPF, BPA and BPAF is 2.91, 3.32 and 4.47, respectively) were located in hydrophobic (deeper) layer of the erythrocyte membrane disturbing its fluidity. Similarly, Duchnowicz and Koter (2002) assessed changes in membrane fluidity of human erythrocytes treated with 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4-dimethylphenol (2,4-DMP). They observed no changes in membrane fluidity at the level of the 5th carbon atom but noted a decrease in the τ_B and τ_C correlation times, which suggested an increase in erythrocyte membrane fluidity at the level of the 16th carbon atom in the fatty acid moiety.

W/S ratio is considered to be a very sensitive parameter showing

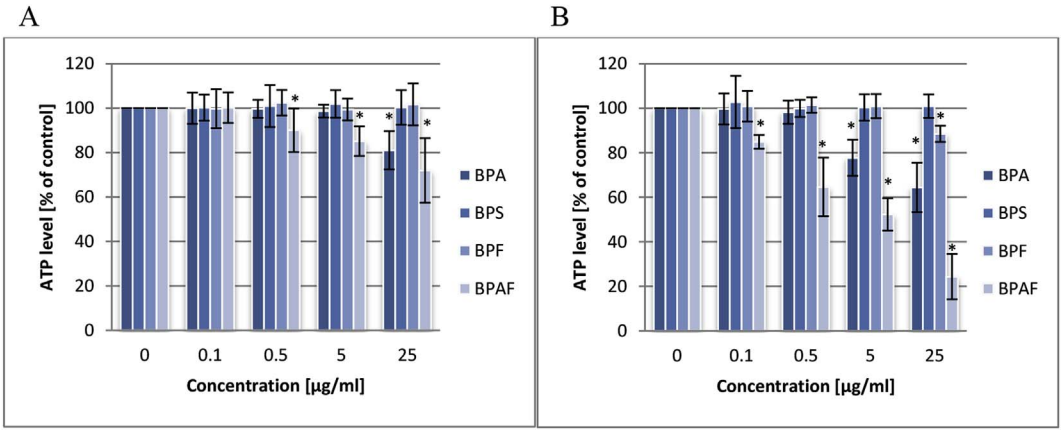

Fig. 6. Changes in ATP level in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.1 to 25 µg/ml for 4 h (A) and 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

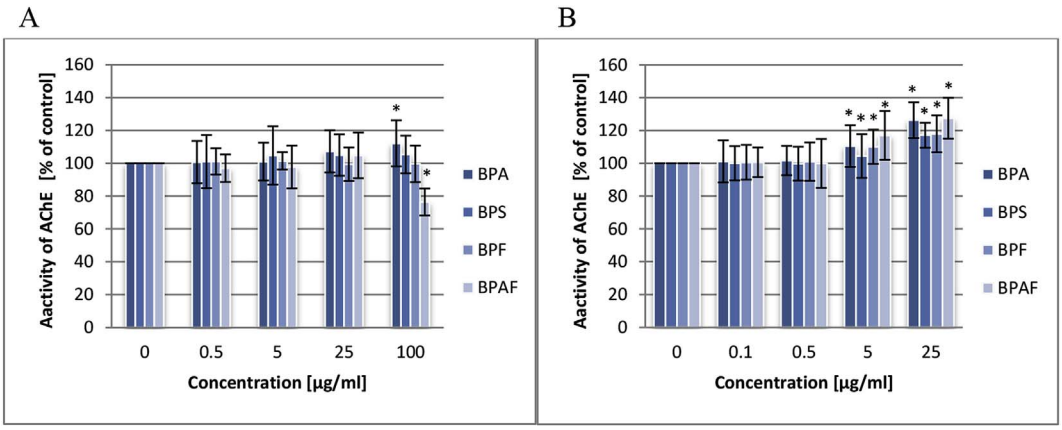

Fig. 7. Changes in AChE activity in human control erythrocytes and the erythrocytes incubated with BPA, BPS, BPF or BPAF in the concentrations ranging from 0.5 to 100 µg/ml for 4 h (A) and from 0.1 to 25 µg/ml for 24 h (B) (*) Significantly different from control ($P < 0.05$); one-way ANOVA and a posteriori Tukey test.

motional and structural behavior of membrane proteins (Gwoździński et al., 2003), while an increase in W/S ratio indicate conformational changes in membrane proteins. Our study showed that all bisphenols studied and BPAF in particular increased this parameter (Fig. 1A and B), which showed that they disturbed membrane protein conformation in human erythrocytes. Changes in W/S ratio may be associated with alterations in the content of sulfhydryl groups of proteins, which are the sites for label attachment (Duchnowicz et al., 2005). We have observed that BPA, BPF and BPAF increased thiol groups level (Fig. 3A and B), which may suggest that bisphenols studied altered protein conformation partly due to the interactions with thiol groups. It has also been shown that products of lipid peroxidation may covalently bind to cysteine, lysine or histidine, which may results in conformational changes of membrane proteins (Pocernich et al., 2001). Our previous study showed that BPA and its analogs increased the level of lipid peroxidation products in human red blood cells (Maćczak et al., 2017), which may explain their ability to alter conformational changes in erythrocyte proteins. Similarly, Duchnowicz et al. (2005) observed conformational changes of proteins and alterations in –SH group level in membrane of human erythrocytes incubated with chlorinated and methylated phenols. In another study, Pawlikowska-Pawlęga et al. (2003) showed that polyphenol quercetin increased W/S ratio in human red blood cells.

Changes in erythrocyte osmotic fragility enables to assess interactions of various xenobiotics with cellular membrane (Mineo et al., 2013). We have observed that bisphenols, and particularly BPA and BPAF increased osmotic fragility of human erythrocytes, which may have been due to disturbing of phospholipid bilayer of membrane, and thus alteration in membrane structure of the cells studied. The results of the recent study (Table 1, Fig. 4) and the study of Maćczak et al. (2015) showed that BPA and BPAF most strongly altered membrane fluidity, caused significant damage to membrane protein and lipids and altered shape of human erythrocytes. Similar results were obtained in the study of Uchendu et al. (2014) who observed an increased osmotic fragility of erythrocytes of rats exposed to pesticides such as chlorpyrifos and deltamethrin.

Damage to membrane proteins may result from direct effect of xenobiotic or its metabolites (e.g. quinones, semiquinones) or indirect influence of reactive oxygen species (ROS) on these macromolecules (Castegna et al., 2002; Bartosz, 2008). We have observed that bisphenols studied, and particularly BPA and BPAF decreased tryptophan fluorescence (Fig. 4A and B), which showed that they caused oxidative damage to proteins in human erythrocyte membrane. Our previous study revealed that BPA and its analogs increased ROS formation, while only BPA and particularly BPAF generated hydroxyl radical in human erythrocytes (Maćczak et al., 2017). It has been proven that hydroxyl radical is mostly involved in oxidative damage to proteins (Bartosz, 2008), therefore the results achieved in the study of Maćczak et al. (2017) may partly explain why BPA and particularly BPAF caused the strongest changes in protein oxidation. In another studies, Bukowska et al. (2008) observed that phenoxyacetic acids, which are the precursors of phenols caused protein damage in human erythrocytes, while Michalowicz et al. (2015) noted that BPA and its analogs induced oxidative damage to proteins in human peripheral blood mononuclear cells (PBMCs).

One of the crucial parameter that influence erythrocyte deformability is its internal viscosity (Słoczyńska et al., 2013; Gwoździński et al., 2017). Our study showed that BPA, BPF and particularly BPAF increased internal viscosity of human red blood cells (data not shown). The observed changes could be associated with alterations in osmolality of the internal fluid of the cell, changes in membrane fluidity or/and alterations in erythrocyte shape. The above mentioned thesis may be confirmed by the finding showing that BPS was the only one among bisphenols studied that did not change the internal viscosity and membrane fluidity of human erythrocytes (Table 1) nor altered the shape of human red blood cells (Maćczak et al., 2015). The number of the research studies evaluating the effect of toxicants on erythrocyte internal viscosity is very small. Bollini et al. (2010) reported that erythrocytes of humans exposed to arsenic present in drinking water had changed internal viscosity, which affected deformability and osmotic fragility of this cell type.

Na^+/K^+ ATPase is one of the most important enzyme located in the erythrocyte membrane. This enzyme plays a crucial role in active transport of various substances across cellular membrane (Duchnowicz et al., 2005). We have observed that all bisphenol studied and particularly BPAF (at higher concentrations) (Fig. 5) depleted the activity of this enzyme. As mentioned above, bisphenols were capable of increasing plasma membrane fluidity at 16th acyl chain carbon depth (Table 1) and also induced lipid peroxidation in human red blood cells (Maćczak et al., 2015). It has been shown that an increase in membrane fluidity (particularly at deeper hydrophobic region) or/and induction of lipid peroxidation may lead to a decrease in Na^+/K^+ ATPase activity (Giraud et al., 1976). In fact, a decrease in the enzyme activity suggest its structural disruption, which may be asscociated with alterations in conformational state (W/S ratio) or/and oxidative damage to proteins, the processes, which were observed in the recent study (Figs. 1 and 4). Moreover, an increase in thiol groups level (Fig. 3) may suggest that BPA and BPAF inhibited the Na^+/K^+ ATPase activity by disulfide bridges destruction. In the study of Duchnowicz et al. (2005) chlorinated phenols and 2,4-DMP have been shown to deplete Na^+/K^+ ATPase activity in human red blood cells, which was due to protein damage and alterations in their conformational state. Similarly, Broncel et al. (2007) reported that flavonoid baicalein decreased Na^+/K^+ ATPase activity in human erythrocytes probably by anchoring to polar heads of the membrane phospholipids.

Changes in ATP level may influence the activity of ATP-dependent enzymes including Na^+/K^+ ATPase (Duchnowicz et al., 2005). It has also been proven that a decrease in ATP level leads to alterations in cell shape, and consequently to a decrease in cell survival (Michałowicz, 2010).

Our recent and previous study showed that BPA, and more strongly BPAF caused significant depletion of ATP level and induced substantial hemolytic changes in red blood cells, while BPS (which did not deplete ATP pull) caused negligible hemolysis in human erythrocytes (Fig. 6, Maćczak et al., 2015). These results may show that different hemolytic potential of bisphenols studied was partly due to different ability of these substances to reduce ATP level in red blood cells. Similarly, Michałowicz et al. (2015) observed that BPA and particularly BPAF most strongly depleted ATP pull, while BPF and BPS in particular decreased ATP level to a lesser extent in human PBMCs, which was correlated with different decrease in viability of this cell type.

Acetylcholinesterase (AChE) present in human red blood cells may be considered as a model AChE in the nervous system. The correlation between AChE inhibition in blood cells and its inhibition in neurons has been well-documented (Bukowska and Hutnik, 2006; Hajjawi, 2012; Sosnowska et al., 2013). The results showed that BPA and its analogs changed AChE activity (Fig. 7). The activity of AChE is modulated by the membrane hydrophobic environment and depends on membrane fluidity and surface charge, while its activity may be changed by xenobiotics, ROS and lipid peroxidation products (Krokosz and Szweda-Lewandowska, 2005; Bukowska and Hutnik, 2006). The strongest changes in AChE activity were noted for BPA and BPAF (Fig. 7), the compounds that most strongly altered membrane fluidity (Table 1), increased lipid peroxidation and generated ROS including hydroxyl radical (Maćczak et al., 2017). We have shown that bisphenols studied caused (mainly) an increase of AChE activity, which may suggest that this enzyme was not damaged, while stimulation of its activity might have been due to the effect of low level of ROS such as hydrogen peroxide as described by Schallreuter et al. (2004). In fact our study showed that AChE activity increased in red blood cells treated with bisphenols studied (particularly BPA and BPAF) after longer incubation time (Fig. 7B). Interestingly, those compounds at lower concentration (25 μg/mL) were capable of inducing slight ROS formation in the erythrocytes incubated for 24 h (data not shown). An increase in AChE activity could have been also associated with alterations in membrane fluidity of the cells studied (Table 1). AChE is located in erythrocyte membrane on phosphatidylinositol, and thus changes in membrane fluidity may lead to stronger exposure of this enzyme outside of the cell, which results in an increase of its activity. We have also noted that BPAF at its highest concentration of 100 μg/ml (after 4 h incubation) decreased AChE activity. This finding may be explained by the ability of this chemical to generate significant amount of ROS including hydroxyl radical and lipid peroxidation products in red blood cells as reported by Maćczak et al. (2017), which can damage to AChE protein. The effect of phenols and its derivatives on erythrocyte AChE activity was observed by Bukowska and Hutnik (2006). They reported that 2,4-DCP, catechol and 2,4-dichlorophenoxyacetic acid decreased the activity of this enzyme.

It must be underlined that BPA, BPF and particularly BPAF induced changes in the parameters studied in human erythrocytes at lower concentrations than those, which have been reported for phenols such as chlorophenols or methylphenols (Duchnowicz and Koter, 2002; Bukowska and Hutnik, 2006). The obtained results also indicate that bisphenols, and particularly BPA and BPAF may potentially disturb function of the erythrocyte membrane in humans occupationally exposed to these substances (He et al., 2009).

5. Conclusions

(1) In this study, for the first time, we have described the mechanism of action of BPA and its analogs, i.e. BPS, BPF and BPAF on human erythrocyte membrane. (2) The compounds studied changed membrane fluidity at hydrophobic region of the plasma membrane, increased internal viscosity and osmotic fragility of the erythrocytes and altered conformational state of membrane proteins. (3) BPA, BPF and BPAF increased thiol groups level, while all bisphenols studied caused oxidative damage to membrane proteins of red blood cells. (4) Bisphenols decreased the activity of Na^+/K^+ ATPase, depleted ATP level (excluding BPS) and changed the activity of AChE. (5). It has been proven that BPAF caused the strongest, while BPS, which is the main substituent of BPA in the manufacture induced the weakest (or none) alterations in the parameters studied. (6) Changes in the parameters examined were observed in the erythrocytes incubated with bisphenols in the concentrations, which may potentially influence humans as a result of occupational exposure.

Conflict of interest

The authors declare that there is no conflict of interest.

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