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„Wpływ stężenia glukozy na właściwości przeciwzapalne resweratrolu w obrębie komórek śródbłonkowych i astrocytów – badania *in vitro* na modelu bariery krew-mózg”.

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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1. WYKAZ STOSOWANYCH SKRÓTÓW (alfabetycznie):

AGEs (ang. Advanced Glycation End Products) – zaawansowane produkty glikacji
ATP – adenozynotrifosforan
BC – przestrzeń mózgowa (ang. brain compartment)
BKM – bariera krew-mózg
ELISA (ang. enzyme-linked immunosorbent assay) – immunoenzymatyczny test fazy stałej, służący do wykrywania i ilościowego oznaczania białek zawartych w badanej próbce
GM-CSF (ang. granulocyte-macrophage colony-stimulating factor) – czynnik stymulujący tworzenie kolonii granulocytów i makrofagów
HSP (ang. heat shock proteins) – białka szoku cieplnego
IFN-γ – interferon gamma
IL-1α – interleukina 1 alfa
IL-1β – interleukina 1 beta
IL-2 – interleukina 2
IL-4 – interleukina 4
IL-6 – interleukina 6
IL-8 – interleukina 8
IL-10 – interleukina 10
IL-12 – interleukina 12
IL-17A – interleukina 17A
LPS – lipopolisacharyd
MC – przestrzeń mikronaczyniowa, (ang. microvascular compartment)
NAD^+ – dinukleotyd nikotynoamidoadeninowy
NF-κB (ang. nuclear factor kappa B) – czynnik jądrowy kappa B
NO – tlenek azotu
OUN – ośrodkowy układ nerwowy
PET (ang. transparent poliester) – przezroczysta membeana poliestrowa
PRR (ang. pattern recognition receptor) – receptory rozpoznające wzorce
RNS (ang. reactive nitrogen species) – reaktywne formy azotu
ROS (ang. reactive oxygen species) – wolne rodniki tlenowe
RSV – resweratrol (3,5,4'-trihydroksy-trans-stilben)
SM (ang. multiple sclerosis) – stwardnienie rozsiane
TLR4 (ang. toll-like receptor 4) – receptor Toll- podobny 4
TNF-α (ang. tumor necrosis factor alpha) – czynnik martwicy nowotworów alfa

2. WPŁYW STĘŻENIA GLUKOZY NA WŁAŚCIWOŚCI PRZECIWZAPALNE RESWERATROLU W OBRĘBIE KOMÓREK ŚRÓDBŁONKOWYCH I ASTROCYTÓW – BADANIA *IN VITRO* NA MODELU BARIERY KREW-MÓZG [STRESZCZENIE]

W związku z rozpowszechnieniem chorób wynikających z pandemii otyłości, w tym cukrzycy typu 2, jak również u osób po zawale serca czy po przebytym udarze, coraz częściej obserwuje się nieprawidłowy poziom glukozy we krwi, zarówno stany hiper- jak i hipoglikemiczne. Należy to uwzględnić oceniając farmakokinetykę leków przechodzących przez barierę krew- mózg (BKM).

Przewlekła hiperglikemia może prowadzić do zwiększonej produkcji wolnych rodników tlenowych (ROS) w astrocytach i komórkach śródbłonka. Nadmiar wolnych rodników może z kolei aktywować czynnik transkrypcyjny NF-κB, który zwrotnie pobudza odpowiedź zapalną, a ponadto zwiększa przepuszczalność bariery krew-mózg (BKM), m.in. dla glukozy. Zaawansowane produkty glikacji (AGE), pojawiające się w wyniku przewlekłej hiperglikemii, mogą także pobudzać układ odpornościowy do produkcji cytokin prozapalnych.

W fizjologii OUN istotną rolę pełni BKM, zabezpieczając mózg przed szkodliwymi czynnikami i umożliwiając selektywny transport substancji z krwi do płynu mózgowo-rdzeniowego. Zdolność do przenikania przez tę barierę zależy od wielu czynników, w tym od stężenia glukozy w surowicy.

Stan zapalny rozwijający się w OUN w wyniku utrzymującego się podwyższonego poziomu glikemii może bezpośrednio uszkadzać neurony, co negatywnie wpływa na funkcjonowanie mózgu, a rozwijająca się encefalopatia cukrzycowa stanowi problem wielu milionów ludzi w skali globalnej. W związku z tym poszukuje się skutecznych metod ograniczenia odpowiedzi zapalnej w OUN.

Udowodniono, że resweratrol - RSV (3,5,4’-trihydroksy-trans-stilben), substancja pochodzenia naturalnego, obecna m.in. w skórce czerwonych winogron, orzechach, w owocach morwy i czarnej porzeczki, posiada właściwości przeciwzapalne, a poza tym zdolność do penetracji przez BKM. Te cechy sprawiają, że RSV potencjalnie spełnia warunki niezbędne do zastosowania w terapii przeciwzapalnej w OUN.

Celem badania była ocena wpływu stanów hipo-, normo- i hiperglikemii w przestrzeni

odpowiadającej naczyniom mikrokrążenia mózgowego (MC, ang. microvascular compartment), na przepuszczalność BKM i działanie przeciwzapalne RSV na poziomie mózgu (BC, ang. brain compartment). Dodatkowo, istotny wydaje się już sam fakt możliwości oceny, czy zmiany poziomu glukozy w istotny sposób zaburzają przenikanie przez BKM substancji o działaniu przeciwzapalnym o masie molowej (M) ok. 230 g/mol (Da), takich jak RSV (izomer *trans*-RSV, M = 228,25 Da), oraz czy modyfikują ich działanie w komórkach astrocytów.

Dane z piśmiennictwa, dotyczące działania RSV w innych układach niż BKM, np. bariera krew-łożysko, pozwalają zakładać, że optymalne środowisko dla aktywności przeciwzapalnych może występować w warunkach normoglikemii, podczas gdy zaburzenia metaboliczne, towarzyszące hiperglikemii je zakłócają. W badaniu opisanym w rozprawie doktorskiej oceniano również wpływ hipoglikemii na te działania RSV.

Do przeprowadzenia eksperymentu został wykorzystany specjalnie utworzony model imitujący w warunkach *in vitro* stosunki panujące po obu stronach BKM, tzn. składał się z dwóch głównych przestrzeni: mikrokrążenia (MC), której powierzchnia styku z BKM pokryta była komórkami śródbłonka, oraz wewnątrzmózgowej (BC- brain compartment), której powierzchnię styku z BKM tworzyły astrocyty. Model BKM zawierał kokultury komórek śródbłonka oraz astrocytów, które były rozdzielone półprzepuszczalną membraną z porami o średnicy 0,4 μm, uniemożliwiającą bezpośredni kontakt pomiędzy różnymi typami komórek.

Utworzono trzy grupy badane: grupa I imitująca warunki hipoglikemii, grupa II – normoglikemii, grupa III – hiperglikemii, przy stężeniach glukozy odpowiednio 40 mg% [2.2 mmol/L], 90 mg% [5.00 mmol/L] oraz 450 mg% [25mmol/L]. Po 24 h inkubacji komórek w warunkach określonego stężenia glukozy dokonano oznaczeń stężenia cytokin IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, INF-γ, TNF-α oraz GM-CSF. Oszacowano również, czy samo stężenie glukozy w MC miało wpływ na różnice w poziomie cytokin prozapalnych w obrębie BC. Następnie w celu wywołania reakcji zapalnej w sposób standardowy (ujednolicony), od strony BC podawano lipopolisacharyd (LPS) w stężeniu 0.2 μM, a po upływie kolejnych 24 h, do MC podawano RSV w stężeniu 50 μM.

Ocena nasilenia stanu zapalnego uwzględniała analizę poziomów cytokin zarówno 12, jak i 36 h po podaniu LPS, a następnie również 24 h po podaniu RSV. Dodatkowo zbadano

stężenie RSV od strony BC, co pozwoliło na określenie stopnia penetracji RSV przez BKM. Wszystkie powyższe oznaczenia przeprowadzono metodą immunoenzymatyczną (ELISA) przy użyciu zestawów kompatybilnych z komórkami ludzkimi, według instrukcji podanych przez producentów. Dodatkowo, oceniano morfologię komórek w ko-hodowli zarówno przed, jak i po barwieniu prostym hematoksyliną i eozyną.

WYNIKI

W porównaniu z normoglikemią, w grupie z obniżonym stężeniem glukozy w MC obserwowano wyższy poziom cytokin prozapalnych w BC. Podobną zależność wykazano w grupie z hiperglikemią, odnotowano wyższe stężenia cytokin w porównaniu z grupą II (normoglikemia). Profil cytokin różnił się w zależności od grupy badanej, co szczegółowo opisano w publikacjach nr 2. i 3., z uwzględnieniem analizy statystycznej.
Po dodaniu RSV od strony naczyniowej BKM zaobserwowano spadek poziomu cytokin we wszystkich grupach badanych. Wyniki badań potwierdzają zdolność przenikania przez BKM oraz działanie przeciwzapalne RSV w BC, przejawiające się obniżeniem poziomu cytokin prozapalnych. Pomimo spadku stężenia cytokin we wszystkich grupach, najbardziej wyraźny efekt przeciwzapalny odnotowano w grupie II, co sugeruje, że normoglikemia stanowi optymalne środowisko dla działań przeciwzapalnych RSV ($p < 0.05$). Ponadto określono stężenia RSV w części mózgowej po 12 h, jak i po 24 h od jego wprowadzenia do MC w trzech grupach badanych. Najwyższe stężenia RSV stwierdzono w środowisku o prawidłowym stężeniu glukozy w MC, a najniższe w przypadku hipoglikemii.

Powyższe wyniki wskazują, że utrzymanie prawidłowej kontroli glikemii może być istotne dla efektywności działania substancji przeciwzapalnych, takich jak RSV. Może to stanowić podstawę do wprowadzenia modyfikacji dawkowania RSV u osób z cukrzycą oraz po przebytym udarze czy zawale serca, w przypadku stanów hipo- i/lub hiperglikemii. Leczenie wspomagające RSV (a także innymi substancjami o podobnych właściwościach przenikającymi przez BKM) znajduje już zastosowanie w chorobach neurodegeneracyjnych, ale dotychczas skuteczności przeciwzapalnej takiej terapii nie wiązano z zaburzeniami glikemii, jakże częstymi u tych pacjentów neurologicznych. Interpretując wyniki, uwzględniono zarówno wady, jak i zalety zastosowanego modelu BKM.

3. THE INFLUENCE OF GLUCOSE CONCENTRATION ON THE ANTI-INFLAMMATORY PROPERTIES OF RESVERATROL IN ENDOTHELIAL CELLS AND ASTROCYTES - *IN VITRO* STUDIES ON THE BLOOD-BRAIN BARRIER MODEL [SUMMARY]

Due to the prevalence of diseases resulting from the obesity pandemic, including type 2 diabetes, as well as in individuals post-heart attack or stroke, abnormal blood glucose levels are increasingly observed, encompassing both hyper- and hypoglycemic states. This should be considered when evaluating the pharmacokinetics of drugs crossing the blood-brain barrier (BBB).

Chronic hyperglycemia can lead to increased production of reactive oxygen species (ROS) in astrocytes and endothelial cells. An excess of free radical species can activate the transcription factor NF-κB, which in turn stimulates the inflammatory response and increases the permeability of the BBB, among other things for glucose. Additionally, advanced glycation end products (AGEs), which appear as a result of chronic hyperglycemia, can stimulate the immune system to produce pro-inflammatory cytokines.

In the physiology of the central nervous system (CNS), the BBB plays a significant role, protecting the brain from harmful factors and enabling selective transport of substances from the blood to the cerebrospinal fluid. The ability to penetrate this barrier is influenced by many factors, such as the level of serum glycemia.

Inflammatory state emerging within the CNS due to sustained hyperglycemia can directly damage neurons, thus adversely affecting brain function. Diabetic encephalopathy, as it develops, poses a significant challenge for millions of individuals worldwide. Consequently, effective methods to mitigate the inflammatory response within the CNS are being sought.

It has been demonstrated that resveratrol (RSV) – a natural substance present, among others, in the skin of red grapes, nuts, mulberries, and black currants- possesses anti-inflammatory properties and, moreover, the ability to penetrate the BBB. These characteristics render RSV potentially suitable for application in anti-inflammatory therapy within the CNS.

The aim of the study was to assess the impact of hypo-, normo- and hyperglycemic states in the space corresponding to cerebral microcirculation vessels (MC- microvascular

compartment) on the permeability of the BBB and the anti-inflammatory action of RSV at the brain level (BC – brain compartment). It seems significant in itself to assess whether glycemic fluctuations significantly disturb the penetration of substances with anti-inflammatory activity with a molar mass (M) of about 230 g/mol (Da) through the BBB, such as RSV (*trans*-RSV isomer, M = 228,25 Da), and whether they modify their action within astrocytes.

Data from the literature regarding the action of RSV in systems other than the BBB, such as the blood-placental barrier, suggest that optimal conditions for anti-inflammatory activity are represented by normoglycemia, while metabolic disturbances accompanying hyperglycemia disrupt it. The study described in the doctoral dissertation also evaluated how this situation presents in hypoglycemia.

A specially created model was used to conduct the experiment, simulating *in vitro* conditions on both sides of the BBB. This model consisted of two main compartments: the microcirculation (MC), whose interface with the BBB was covered with endothelial cells, and the brain compartment (BC), whose interface with the BBB was formed by astrocytes. The BBB model included cocultures of endothelial cells and astrocytes, which were separated by a semipermeable membrane with pores of 0.4 μm in diameter, preventing direct contact between the different cell types.

Three experimental groups were created: Group I simulating hypoglycemic conditions, Group II simulating normoglycemic conditions, and Group III simulating hyperglycemic conditions, with glucose concentrations of 40 mg% [2.2 mmol/L], 90 mg% [5.00 mmol/L], and 450 mg% [25 mmol/L], respectively. After 24 h of cell incubation under the specified glucose concentrations, measurements were taken for the concentrations of cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, INF-γ, TNF-α, and GM-CSF. It was also assessed whether the glucose concentration in MC alone affected differences in pro-inflammatory cytokine levels within BC. Subsequently, to induce an inflammatory response in a standardized manner, LPS was administered to BC at a concentration of 0.2 μM, and after additional 24 h, RSV was administered to MC at a concentration of 50 μM.

The assessment of the severity of the inflammatory state included an analysis of cytokine levels at both 12 and 36 h after LPS administration, followed by 24 h after RSV administration. Additionally, the concentration of RSV on the BC side was examined, allowing for the determination of the degree of RSV penetration through the BBB. All the

above assays were conducted using the enzyme-linked immunosorbent assay method (ELISA) with kits compatible with human cells, according to the manufacturers' instructions. Moreover, the morphology of cells in co-culture was evaluated both before and after staining with hematoxylin and eosin.

RESULTS

In comparison to normoglycemia, the group with reduced glucose concentrations in the MC exhibited higher levels of pro-inflammatory cytokines in the BC. A similar association was observed in the hyperglycemic group, where greater cytokine concentrations were noted compared to Group II (normoglycemia). The cytokine profile varied depending on the study group, as detailed in publications No. 2 and 3, with statistical analysis taken into account.

Research findings confirm the permeation capability of BBB and the anti-inflammatory action of RSV in BC, manifested by the reduction of pro-inflammatory cytokine levels. Despite the decrease in cytokine concentrations across all groups, the most pronounced anti-inflammatory effect was observed in group II, indicating that normoglycemia provides an optimal environment for the anti-inflammatory actions of RSV ($p<0.05$). Moreover, the concentration of RSV in the BC was determined, both 12 and 24 h after its introduction into the MC in the three experimental groups. The highest concentration of RSV was detected in the environment with normal glucose concentration in the MC, and the lowest in the environment with hypoglycemia.

The above results indicate that maintaining proper glycemic control may be significant for the effectiveness of anti-inflammatory agents such as RSV. This may provide a basis for adjusting the dosage of RSV in individuals with diabetes as well as those who have experienced a stroke or heart attack, in cases of hypo- and/or hyperglycemia. Adjunctive therapy with RSV (as well as other substances with similar BBB permeating properties) is already being used in neurodegenerative diseases, but until now, the anti-inflammatory efficacy of such therapy has not been associated with glycemic disturbances, which are common in these neurological patients.

4. WSTĘP

4.1. Uzasadnienie wyboru tematu i podjęcia badań

Znaczący rozwój medycyny i technologii medycznej w ciągu ostatnich kilku dekad doprowadził do wzrostu liczby osób starszych w populacji ogólnej. W związku z tym coraz więcej ludzi może cierpieć z powodu chorób neurodegeneracyjnych, takich jak choroba Alzheimera czy choroba Parkinsona. Wiek jest ponadto istotnym czynnikiem ryzyka udaru mózgu, zarówno niedokrwiennego, jak i krwotocznego. Należy pamiętać, że stan zapalny odgrywa wiodącą rolę w patomechanizmie nie tylko chorób *stricte* zapalnych w ośrodkowym układzie nerwowym (OUN), takich jak np. stwardnienie rozsiane (SM), ale także wiąże się z progresją wymienionych powyżej chorób neurodegeneracyjnych [1-6]. Wyniki najnowszych badań wspierają hipotezę, iż jedną z opcji leczenia neuroprotekcyjnego może być stosowanie związków polifenolowych pochodzenia roślinnego, takich jak resweratrol (RSV), który posiada udokumentowane działanie przeciwzapalne oraz antyoksydacyjne [7-10]. Co istotne, ze względu na swoją niską masę cząsteczkową, wynoszącą dla wykazującego aktywność biologiczną izomeru *trans* 228,25 Da i rozpuszczalność w tłuszczach, RSV posiada zdolność do przenikania przez barierę krew-mózg (BKM). To sprawia, że mógłby być potencjalnie wykorzystany w ograniczaniu stanu zapalnego w OUN. Przeciwzapalne i przeciwutleniające właściwości RSV są związane m.in. z jego zdolnością do aktywacji sirtuin, hamowania szlaku sygnalizacyjnego czynnika jądrowego kappa B (NF- κB), jak również aktywacji autofagii zamiast apoptozy [11-14]. Pomimo jeszcze nie w pełni wyjaśnionych mechanizmów działania, potwierdzono w licznych badaniach korzystny efekt działania RSV na procesy poznawcze i pamięć [15-18]. W pierwszej publikacji, włączonej do cyklu rozprawy doktorskiej, przeprowadziłam przegląd systematyczny badań eksperymentalnych i klinicznych, w którym oszacowałam potencjalne korzyści ze stosowania RSV w zapobieganiu i leczeniu choroby Alzheimera, m.in. w związku z ograniczaniem nasilenia stanu zapalnego w OUN.

Poza starzeniem się społeczeństw, istotnym problemem zdrowotnym jest wzrost częstości występowania chorób związanych z pandemią otyłości, w tym zaburzeń tolerancji glukozy (insulinooporności) i cukrzycy typu 2. Ponieważ komórki nerwowe preferencyjnie wykorzystują glukozę do uzyskania energii, wahania poziomu glukozy (zarówno hiperglikemia, jak i hipoglikemia), mogą wpływać na procesy metaboliczne w OUN,

prowadząc do indukcji odpowiedzi zapalnej, zarówno w wyniku zwiększonej produkcji wolnych rodników tlenowych (ROS), jak i biosyntezy zaawansowanych produktów glikacji (AGE) [19-24]. Przewlekły stan zapalny może zaburzać integralność i zwiększać przepuszczalność BKM, co z kolei może promować penetrację potencjalnie toksycznych lub immunogennych substancji do OUN, powodując uszkodzenie neuronów [25-27]. Powyższe zaburzenia zostały wykryte m.in. w chorobie Alzheimera, chorobie Parkinsona, zapaleniu mózgu, zapaleniu opon mózgowo-rdzeniowych, stwardnieniu rozsianym (SM) i neurosarkoidozie [28-32].

4.2. Wpływ stężenia glukozy na indukcję stanu zapalnego

Prawdopodobieństwo wystąpienia reakcji zapalnej i stopień jej nasilenia stosunkowo trudno przewidzieć w cukrzycy, gdyż, jak wykazano w przeprowadzonych badaniach, profil cytokin w OUN zależy od stężenia glukozy w układzie odpowiadającym naczyniom mikrokrążenia mózgowego (MC). Przejściowe, wysokie stężenie glukozy we krwi nie są szkodliwe dla neuronów, ponieważ przepływ glukozy jest ściśle kontrolowany przez zdrowy układ BKM, ale przedłużone okresy hiperglikemii u pacjentów z cukrzycą zaburzają integralność BKM i prowadzą do wytwarzania ROS w astrocytach, głównie ze względu na przyspieszony proces glikolizy tlenowej, a następnie kolejnych reakcji w cyklu kwasu cytrynowego, gdzie dochodzi do przekształcenia utlenionego dinukleotydu nikotynoamidoadeninowego (NAD^+) w jego zredukowaną formę NADH [33]. Należy podkreślić, że ani NAD^+ ani NADH nie przechodzą przez błonę mitochondrialną, a zatem wyczerpanie mitochondrialnego NAD^+ nie może być uzupełnione przez zwiększony napływ NAD+ z innych kompartmentów komórkowych, a nadmiar NADH nie może być eliminowany z mitochondrium poprzez dyfuzję prostą [34]. Elektrony przenoszone z NADH są przekazywane do łańcucha transportu elektronów w celu wytworzenia ATP, ale proces ten jest nieskuteczny, gdy poziom ATP w komórce jest już wysoki. W takich okolicznościach NADH może przenosić elektrony nieenzymatycznie do cząsteczek tlenu, co prowadzi do produkcji ROS [35]. Wysokie poziomy ROS w astrocytach mogą wywołać aktywację NF-κB, prowadzącą do zapalenia i wzrostu przepuszczalności naczyniowej [36]. Wówczas napływ glukozy do OUN przestaje być precyzyjnie kontrolowany, co prowadzi do zwiększonego przenikania glukozy do neuronów i wywołuje w nich odpowiedź zapalną. Ponadto, uszkodzenie mitochondrialnego DNA wskutek zwiększonego stężenia ROS może indukować produkcję białek szoku cieplnego, takich jak HSP-60 [37]. Niektóre cząsteczki

HSP-60 cechuje zdolność przenoszenia się do innych regionów komórki, takich jak błona komórkowa i cytoplazma, gdzie mogą aktywować receptory rozpoznające wzorce (PRR), które z kolei inicjują odpowiedź zapalną związaną z odpornością wrodzoną [38]. Dodatkowo, odporność wrodzona może być aktywowana przez AGE [39].

Mózg nie produkuje glukozy ani nie przechowuje znacznych rezerw glikogenu wewnątrz astrocytów, dlatego jego funkcjonowanie zależy od stałego napływu glukozy z krwioobiegu [20]. W rezultacie hipoglikemia prowadzi do wyczerpania rezerw energetycznych mózgu, indukując zaburzenia poznawcze, które finalnie mogą doprowadzić do śpiączki, a nawet martwicy komórek nerwowych [40]. Istnieje prawdopodobne powiązanie pomiędzy powtarzającymi się epizodami głębokiej hipoglikemii, w tym jatrogennej jako niepożądanego efektu działania leków obniżających stężenie glukozy we krwi, a trwałym upośledzeniem poznawczym [41]. Podczas gdy dokładny mechanizm pozostaje nieznany, udowodniono, że hipoglikemia doprowadza do wzrostu stężenia cytokin prozapalnych i leukocytozy, co wskazuje na potencjalny związek między hipoglikemią a odpowiedzią zapalną [42].

4.3. Potencjalne działanie przeciwzapalne RSV w OUN

Stan zapalny w OUN wykryto m.in. w chorobach neurodegeneracyjnych (choroba Alzheimera, choroba Parkinsona), SM i u pacjentów po udarze mózgu [2-5, 43]. Przewlekłe zapalenie w OUN przyczynia się do progresji chorób i uszkodzenia neuronów, co stanowi istotny problem zdrowotny i dotyczy milionów ludzi na świecie [43]. Powyższe uzasadnia prowadzenie badań naukowych w celu znalezienia skutecznych leków i strategii terapeutycznych ograniczających szkodliwy wpływ przewlekłego zapalenia w OUN.

RSV, to naturalnie występujący polifenol, który został wykryty w niektórych organach roślin wyższych, m.in., w skórce czerwonych winogron, w owocach jagodowych, orzeszkach ziemnych i kakao [44,45]. RSV posiada zdolność modulowania różnych szlaków sygnalizacyjnych biorących udział w zapaleniu, poprzez hamowanie aktywacji czynników transkrypcyjnych (np., NF- κB) lub poprzez aktywację sirtuin [46,47].

Dodatkowo jako środek antyoksydacyjny RSV może neutralizować ROS oraz reaktywne formy azotu (RNS), takie jak tlenek azotu (NO), dzięki obecności grupy hydroksylowej– OH oraz chronić neurony poprzez zwiększenie ekspresji enzymów antyoksydacyjnych, takich jak dysmutaza ponadtlenkowa, katalaza czy peroksydaza glutationowa [48-51].

Badania kliniczne wykazały, że RSV jako jeden z nielicznych polifenoli, cechuje zdolność do przenikania przez BKM [52]. Ta właściwość, jak i udowodnione działanie przeciwzapalne powodują, że RSV może okazać się obiecującym związkiem w badaniach nad skutecznym leczeniem chorób OUN. W cyklu badań włączonych do rozprawy doktorskiej (publikacje nr 2. i 3.) dokonano oceny działań przeciwzapalnych RSV w zależności od stężenia glukozy w MC na modelu BKM w warunkach *in vitro.*

4.4. Uzasadnienie połączenia publikacji w jeden cykl

Publikacje zawarte w cyklu rozprawy doktorskiej stanowią spójną całość, akcentując ważny aspekt chorób neurodegeneracyjnych, a mianowicie współistniejący stan zapalny, który mógłby zostać ograniczony dzięki zastosowaniu RSV. W dwóch publikacjach (prace oryginalne) przedstawiono wyniki badań, w których dokonano oceny wpływu różnych stężeń glukozy w przestrzeni odpowiadającej MC na profil cytokin w przestrzeni BC. W artykułach oszacowano również przepuszczalność BKM dla RSV i działanie przeciwzapalne RSV w zależności od poziomu glukozy w MC. Wszystkie badania zostały przeprowadzone w warunkach *in vitro*, na specjalnie przygotowanym modelu BKM. W publikacji pt. „*The Role of Glucose Concentration and Resveratrol in Modulating Neuroinflammatory Cytokines: Insights from an In Vitro Blood-Brain Barrier Model"(nr 2.),* analizowano zmiany profilu cytokin pod wpływem RSV przy różnych stężeniach glukozy, w odniesieniu do interleukiny10 (IL-10), interleukiny 12 (IL-12), interleukiny 17A (IL-17A), czynnika martwicy nowotworów alfa (TNF-α), interferonu gamma (IFN- γ) oraz czynnika stymulującego wzrost kolonii granulocytów i mikrofagów (GM-CSF). W kolejnym artykule pt. „*Anti-Inflammatory Action of Resveratrol in the Central Nervous System in Relation to Glucose Concentration-An In Vitro Study on a Blood-Brain Barrier Model" (nr 3.*), kontynuowano powyższą analizę w odniesieniu do innych cytokin, a mianowicie: interleukiny 1 alfa (IL-1α), interleukiny 1 beta (IL-1β), interleukiny 2 (IL-2), interleukiny 4 (IL-4), interleukiny 6 (IL-6) oraz interleukiny 8 (IL-8). Ponadto, w publikacji przeglądowej pt. „*Review of beneficial effects of resveratrol in neurodegenerative diseases such as Alzheimer's disease"(nr 1.),* przedstawiono i skomentowano charakterystykę potencjalnie korzystnego działania RSV w chorobach neurodegeneracyjnych, takich jak choroba Alzheimera, na podstawie przeglądu badań eksperymentalnych i klinicznych przeprowadzonych w ciągu ostatnich 10 lat (przegląd systematyczny).

5. ZAŁOŻENIA I CEL PRACY

5.1. Cel i informacje ogólne

Celem przeprowadzonych badań było ustalenie czy różne stężenia glukozy, odpowiadające stanom hipo-, normo- i hiperglikemii, w przestrzeni odpowiadającej naczyniom mikrokrążenia mózgowego (MC):

– oddziałują na profil cytokin prozapalnych w przestrzeni mózgowej (BC)
– wpływają na przepuszczalność BKM dla RSV i jego działanie przeciwzapalne, przejawiające się spadkiem stężeń cytokin prozapalnych w BC.

Badania zostały przeprowadzone między styczniem 2021 r. a marcem 2022 r. w Centrum Badań Przedklinicznych i Technologii Warszawskiego Uniwersytetu Medycznego (CePT). Zgodnie z najnowszą wersją Deklaracji Helsińskiej, ponieważ prace badawcze zostały przeprowadzone na dostępnych komercyjnie liniach komórkowych, bez żadnych implikacji klinicznych, nie było wymagań odnośnie uzyskania dodatkowej zgody. Niemniej jednak, badanie zostało zgłoszone i zaakceptowane przez Komisję Bioetyczną Warszawskiego Uniwersytetu Medycznego w dn. 14.12.2020 r. (nr AKBE220/2020).

5.2. Założenia pracy

Utworzono specjalny model BKM, zawierający kokulturę komórek śródbłonka i astrocytów, rozdzieloną przezroczystą membraną poliestrową (PET, ang. transparent poliester), odpowiadającą błonie półprzepuszczalnej z porami o średnicy 0,4 μm, uniemożliwiającej bezpośredni kontakt między różnymi typami komórek. Linię komórkową śródbłonka ludzkiego hCMEC/D3, zawierającą komórki pochodzące ze śródbłonka naczyniowego mózgu, zakupiono od firmy Cederlane Cellutions Biosystems (Burlington, ON, Kanada, nr katalogowy #CLU512). Według producenta, linia ta może być używana w badaniach komórkowych i molekularnych układu nerwowego lub jako model pojedynczych komórek BKM. Celem uzyskania 100% konfluencji komórek endotelialnych użyto dedykowanego medium EBM-2 (Merck KGaA, Darmstadt, Niemcy, Sigma Aldrich, nr kat. #C-22211). Astrocyty ludzkie pochodzące ze zdrowej tkanki mózgowej, również dostępne komercyjnie, zakupiono w firmie Thermo Fisher Scientific-

Gibco TM (nr kat. #N7805100), wraz z GibcoTM Astrocyte Medium (nr kat. #A1261301). Proliferacja komórek śródbłonka i astrocytów trwała odpowiednio 3 i 4 dni, w specjalnych butelkach hodowlanych. Po uzyskaniu 100% konfluencji, zawiesina astrocytów została dodana do dołków (studzienek) hodowlanych na płytkach pokrytych matrycą podstawową, a po 24 h wymieniono medium. Następnie wkłady zostały pokryte kolagenem typu I i umieszczone w dołkach. Po zastygnięciu kolagenu do każdego wkładu wprowadzono zawiesinę komórek śródbłonka, a po kolejnych 24 h, roztwór został wymieniony na nowy z wcześniej ustalonym stężeniem glukozy. Otrzymany model BKM zawierał astrocyty reprezentujące BC oraz komórki śródbłonka reprezentujące MC.

W badaniu utworzono trzy grupy badane, różniące się stężeniami glukozy w MC, odpowiadające stanom: hipoglikemii – grupa I, normoglikemii – grupa II i hiperglikemii – grupa III (stężenia molowe odpowiednio: 2.2, 5.0 i 25.0 mmol/l). Wstępnie założono, iż nieprawidłowy poziom glukozy w przestrzeni MC w grupie I i III spowoduje wzrost poziomu cytokin w BC (pomiar po 24 h) w stosunku do grupy II, w której stężenie glukozy było prawidłowe.

Celem uzyskania powtarzalnych (standardowych) warunków natężenia reakcji zapalnej, do płytek hodowlanych tworzących BKM po stronie BC wprowadzono roztwór LPS, o stężeniu 0.2 μM. Po 12 h i 36 h za pomocą testu ELISA badano stężenia cytokin w przestrzeni BC w trzech grupach badanych, z różnymi stężeniami glukozy w przestrzeni MC. Przyjęto założenie, że LPS spowoduje większy wzrost poziomu cytokin prozapalnych w grupach I oraz III, w stosunku do grupy II, zarówno po 12 h, jak i po 36 h. Na tym etapie badań oceniano również, czy użycie LPS w grupach o różnym stężeniu glukozy wpłynie na morfologię astrocytów. Wstępnie założono możliwość różnic morfologii astrocytów w różnych grupach badanych po zastosowaniu LPS. Ocena morfologii komórek była uzupełniona barwieniem przyżyciowym astrocytów w teście wykluczenia (ekskluzji) z użyciem błękitu trypanu, w celu określenia ich żywotności. Celem tych procedur było ustalenie, czy istnieją przesłanki do zastosowania technik komputerowej analizy obrazu z morfometrią.

W kolejnym etapie badania, po 36 h od podania LPS do przestrzeni BC, do każdej próbki w MC podawano roztwór RSV o stężeniu 50 μM, celem oceny wpływu RSV na ograniczenie odpowiedzi zapalnej w przestrzeni BC. Korzystając z zestawu Multi-Analyte

ELISArray, dokonano pomiaru stężeń cytokin w próbkach pobranych z przestrzeni BC, testując hipotezę, iż środowisko normoglikemii jest optymalne dla działania przeciwzapalnego RSV, przejawiającego się najwyższym spadkiem stężeń cytokin prozapalnych w grupie II. Ponadto za pomocą testu ELISA określono i porównano stężenia RSV w przestrzeni BC pomiędzy badanymi grupami. Na podstawie przeglądu aktualnego piśmiennictwa założono, że przenikanie RSV do przestrzeni BC będzie największe w grupie III, gdyż środowisko hiperglikemiczne może powodować uszkodzenia i zaburzenia integralności BKM, a zatem może ułatwiać przechodzenie substancji do przestrzeni mózgowej w większym stopniu, w porównaniu do grupy z prawidłowym i obniżonym stężeniem glukozy.

Schemat przedstawiający etapy tworzenia modelu BKM w warunkach *in vitro* oraz przeprowadzonego badania został przedstawiony w publikacji nr 2: „*The Role of Glucose Concentration and Resveratrol in Modulating Neuroinflammatory Cytokines: Insights from an In Vitro Blood-Brain Barrier Model"* (Figure 3).

Do weryfikacji modelu badawczego BKM i umożliwienia prawidłowej interpretacji wyników niezbędne było utworzenie odpowiednich grup kontrolnych. W pierwszym etapie badania utworzono grupę kontrolną w postaci ko-hodowli komórek endotelialnych z astrocytami w medium nie zawierającym glukozy. Podczas drugiego etapu badania (wprowadzenie LPS) grupa kontrolna zawierała podstawowe medium z 0.2 µM roztworem LPS, również pozbawione glukozy. W ostatnim etapie badania utworzono grupę kontrolną z podstawowym medium zawierającym 0.2 µM roztworu LPS oraz 50 µM roztworu RSV, bez dodatku glukozy.

Wszystkie pomiary ilościowe techniką ELISA powtórzono sześciokrotnie, po czym obliczono wartości średnie (wyniki zostały przedstawione w rozdziałach pt. „*Results"* w publikacjach nr 2. i 3. zawartych w rozprawie doktorskiej), a następnie dokonano analizy statystycznej.

Analiza statystyczna uzyskanych danych została przeprowadzona za pomocą języka programowania R i jego wbudowanych funkcji (projekt R do obliczeń statystycznych, wersja 4.0.5), za pomocą testu U Manna-Whitneya. Wyniki uznano za istotne statystycznie, gdy wartości *p* wynosiły poniżej 0.05 ($p < 0.05$). Opis zastosowanych metod statystycznych w poszczególnych etapach badania zestawiono w rozdziałach „*Results"* w obu pracach oryginalnych zamieszczonych w niniejszej rozprawie.

6. KOPIE OPUBLIKOWANYCH PRAC

Rozdziały 6.1.-6.3. stanowią cykl publikacji wchodzących w skład prezentowanej rozprawy doktorskiej.

6.1.

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Review article

Review of beneficial effects of resveratrol in neurodegenerative diseases such as Alzheimer's disease

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ABSTRACT

Purpose: The prevalence of Alzheimer's Disease is rising, in part due to increase in the medium age of residents in developed countries. The aim of the study has been to determine whether resveratrol (RSV) can be effective in the prevention or treatment of Alzheimer's Disease, providing its antioxidant, anti-inflammatory, and SIRT1-activating properties.

Methods: A systematic review of some experimental and clinical studies has been made. The eligibility criteria have comprised: maximum 10 years passed from the study publication, geographical diversity of the studies performed, and - as much as possible - pertaining of the reviewed study results both to animal models of AD, and to humans.

Results: After the final assessment of the eligibility criteria, 96 research studies have been included in the review. Overall results suggest that RSV can be effectively used in the prevention of AD, especially in reference to its familial forms with an early onset. At the same time, efficacy of RSV in the treatment of AD needs further studies, aimed at: improving its transport through the blood-brain barrier (BBB), performing prospective clinical *in vivo* trials on large groups of patients, and determining the optimal RSV dosage.

Discussion: Providing RSV mechanisms of action, inhibitory in reference to many pathomechanisms of AD, it seems very likely that RSV could be effective in AD prevention. The main limitations referring to such presumption include: limited permeability of BBB to RSV, and scarcity of clinical studies on RSV pertaining to large groups of humans.

1. Introduction

1.1. Rationale for the review

Remarkable development of medicine and medical technology has led to the increased number of elderly people in the general population over the last few decades. It is estimated that about 11% of the world population has reached the age of 60 and this percentage may increase to 22 in 2050 [1]. Thus, more people are likely to be affected by age-related diseases, including those associated with neurodegeneration, such as Alzheimer's disease (AD) or Parkinson's disease (PD). The number of humans affected by neurodegenerative diseases may double every twenty years, unless effective treatment is implemented [2]. Since the exact pathomechanism of those diseases remains incompletely understood, it is difficult to outline the effective treatment. Nevertheless, some available evidence supports the hypothesis that one of the possible options of neuroprotective treatment may comprise the use of plant-derived polyphenolic compounds, such as RSV This substance has some well-documented anti-tumour, cardio-protective, anti-bacterial, anti-inflammatory and anti-aging properties [3]. Furthermore, RSV is capable of crossing the blood brain barrier (BBB), so it can act effectively on the central nervous system (CNS) [4]. Possible efficacy of RSV in AD prevention and treatment can be presumed on the basis of its antioxidant, anti-inflammatory, and SIRT1-activating properties.

1.2. Objectives

The aim of this paper is to analyze the possible benefits of RSV use in the neurodegenerative diseases, on the basis of current research. Insight into the mechanisms of inflammation in AD and PD may help to create some novel therapeutic strategies, possibly targeting the inflammatory process. We aimed to determine whether sufficient evidence exists that RSV can counteract AD pathomechanisms and significantly delay its onset. We also aimed to check how many research

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studies completed so far in this field pertain to humans, and, if so, what effects have been observed.

1.3. Protocol

A systematic review of some experimental and clinical studies has been made. The eligibility criteria included: maximum 10 years passed from the study publication, geographical diversity of the studies performed, and - as much as possible - the reviewed study results pertain both to animal models of AD, and to humans. PubMed database has been searched though, using such conjunctions of keywords as 'resveratrol and neurodegeneration', 'resveratrol and neuroprotection', 'resveratrol and oxidative stress', 'resveratrol and inflammation', 'resveratrol and Alzheimer's disease', 'Alzheimer's disease and etiology', 'Alzheimer's disease and pathomechanism'. These keywords have been searched for in titles, or, when nothing found, in title/abstract sections. Subsequently, the most relevant studies have been included in this review. The relevance assessment has been made on the basis of the number of issues relevant for the aim of our review. The main issues addressed included: 1 - current knowledge about RSV sources, chemical properties, and actions, 2 - current knowledge about AD pathomechanisms. PRISMA flow diagram for study selection in our review is presented in Fig. 1.

2. Review

2.1. What is resveratrol and where it can be found?

RSV is one of the polyphenols that can be found in some organs of higher plants, including leaves, roots, stalks, seeds and whole fruit. According to some research studies, various levels of polyphenolic compounds have been reported in different parts of the plants [5]. The production of polyphenolic compounds in plants is upregulated by exposure to UV radiation, fungal/bacterial infections or oxidative stress. One of these compounds, RSV, is produced both in the epidermal leaf and in the grape peel in response to infection with grey mold (*Botrytis cinereal*), grape downy mildew (*Plasmopara viticola*) and black bread mold (*Rhizopius nigricans*) [6]. In addition, polyphenols can protect plants from adverse environmental conditions such as water deficiency, low temperature, or tissue damage.

RSV (3,4',5-trihydroxystilbene) was first isolated from *Veratrum grandiflorum*, or white hellebore plant, in 1940s [7]. One of the best natural sources of RSV are some red grape parts, with fresh grape peel containing approximately 50–100 mg per 1 g, which represents 5–10%

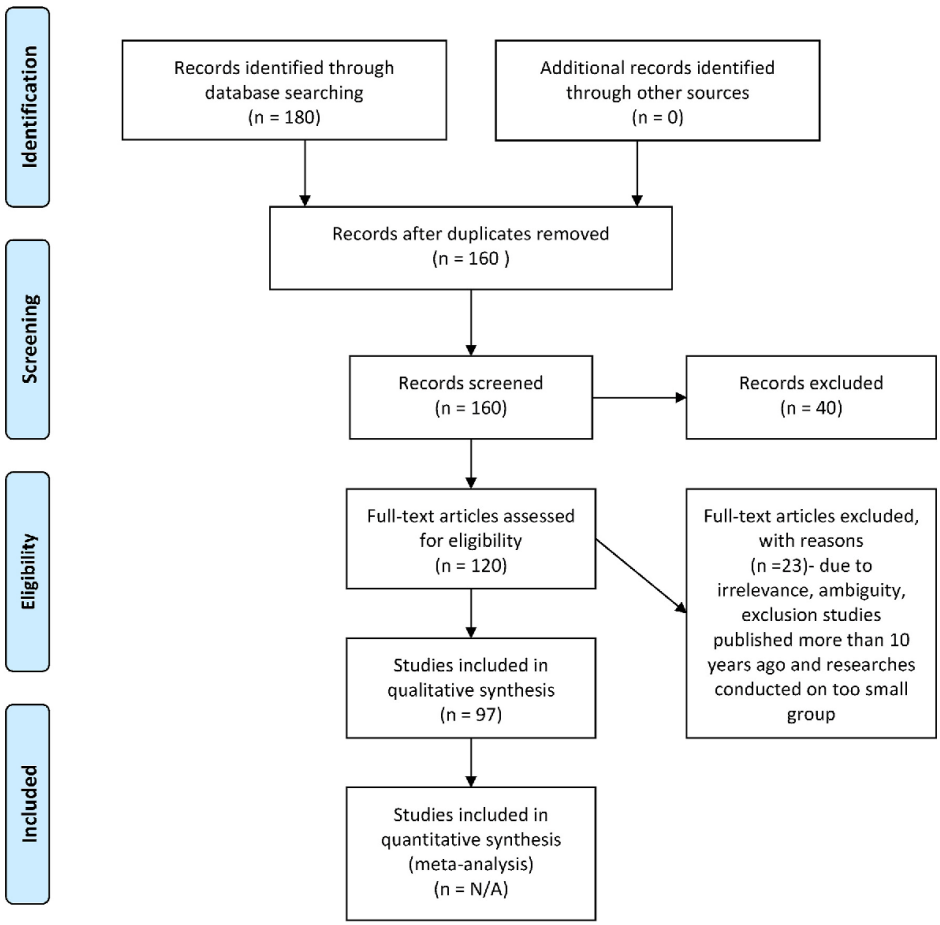

Fig. 1. PRISMA flow diagram for study selection. Adapted from: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6 (7): e1000097. https://doi.org/10.1371/journal.pmed1000097.

Table 1
Sources of RSV and their estimated concentration.

Sources of resveratrol	Resveratrol concentration
Bilberries	~16 ng/g
Blueberries	~32 ng/g
Cranberry juice	~0.2 mg/L
Cocoa powder	~1.85 μg/g
Dark chocolate	~0.35 μg/g
Milk chocolate	~0.10 μg/g
Peanut	~0.02–1.92 μg/g
Pistachios	~0.09–1.67 μg/g
Red grape juice	~0.50 mg/L
Red wine	0.02–14.3 mg/L
White grape juice	~0.05 mg/L
White wine	< 0.1–2.1 mg/L

of their biomass. RSV can also be found in low quantities in wine (0.3–7 mg of L-1 aglycones and 15 mg of l-1 glycosides in red wine) [8].

Many fruits, including all berry fruits (mulberry, cranberry, tufted blueberry, lingonberry, inedible blueberry, bilberry, blackcurrant, strawberries, raspberries, deerberries, partridgeberries), jackfruits and apples, also contain RSV. RSV also has been found in peanuts, pistachios, cocoa and chocolate [9–11].

The richest sources of RSV are roots of *Reynoutria japonica (Polygonum cuspidatum Ko-jo-kon)*, mainly cultivated in China and Japan. In those countries, *Itadori tea* is another rich source of RSV. Finally, this polyphenol can be found in a wide variety of flowers and leaves, including gnetum, white hellebore, corn lily, butterfly orchid tree, eucalyptus, spruce, poaceae, rhubarb and scots pine [12,13]. Table 1 presents main dietary sources of RSV and their estimated concentration.

Due to the presence of a double bond between two carbon atoms connecting the two aromatic rings, they can occur in the form of two isomers – cis and trans. Both isomers can be found in the peel of red grapes and thus in red wine. The trans isomer, which is a predominant form, is much better known. The cis form is produced as a result of the isomerization of the *trans*-RSV form, or disintegration of RSV polymer molecule in such conditions as fermentation process of grape peels, exposure to UV rays and high environmental pH [14].

In humans, ingested RSV is absorbed into the small intestine, subsequently being rapidly metabolized in hepatocytes by cytochrome P450. It is converted to *piceatannol* and *trihydroxystilbene* [15]. In the bloodstream, it is converted to sulphite derivatives, which are finally excreted with urine and feces [16]. Clinical trials have indicated that RSV can easily penetrate the blood-brain barrier (BBB).

2.2. Why it is thought that RSV may be effective against AD?

In humans, polyphenols exhibit anti-inflammatory, antioxidant, and antiangiogenic properties. They can neutralize some molecules harmful to proteins, DNA and RNA - including reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipid radicals. Chemical structure of RSV molecule is presented in Fig. 2.

Polyphenolic compounds contain an aromatic ring with a hydroxyl group –OH [17]. Thanks to the presence of the hydrophilic –OH group,

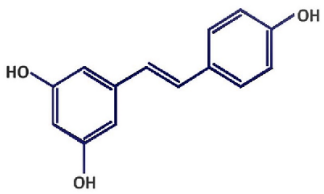

Fig. 2. Structural formula of resveratrol.

polyphenols can react with biologically active compounds, such as enzymes, using hydrogen bonds, which can account for plant protection from microbes. In human organisms, it is exactly the presence of hydroxyl groups that makes polyphenols capable of neutralizing ROS and RNS, such as peroxynitrite and nitric oxide (NO), as well as protein and lipid radicals [18]. Phenols (Ar-OH) are known to reduce the rate of organic matter oxidation by transferring a hydrogen atom from their –OH groups to the radicals containing an ROO* group, most likely through a concerted transfer of one proton and one electron onto the two oxygen atoms - O–H—O* (proton-coupled electron transfer- PCET mechanism) [19]. It has been shown that removal of hydroxyl groups from such compounds results in the loss of their free radicals-neutralizing capability [20].

In addition, an aromatic hydrophobic ring is capable of absorbing UV radiation in the range of 270–320 nm, thus protecting the DNA of plant-derived cells from damage.

2.3. Beneficial actions of RSV in humans

RSV, which naturally occurs in the non-flavonoid fraction of polyphenolic compounds, is reported to have anti-inflammatory and immunomodulatory properties in humans. However, the exact mechanisms of its actions remain unclear.

In their research study, Wiedemann et al. [21], focused on the immunomodulatory effects of RSV towards BV-2 microglial cells by means of transcriptome analysis, using DNA-microarrays and selected qRT-PCRs as methods. The effects of RSV on microglia morphology, phagocytosis and migration have been analyzed and its neurotoxicity on 661 W photoreceptors has been estimated by quantification of caspase 3/7 levels. It has been found that RSV effectively blocks gene expression of a broad spectrum of lipopolysaccharide (LPS)-induced pro-inflammatory molecules, including cytokines and complement proteins. The effects described above are accompanied by a potent inhibition of LPS-induced NO secretion and microglia-mediated apoptosis in 661 W photoreceptor cultures. Moussa et al. [22] found that RSV and other STACs can provide a neuroprotective effect in people suffering from AD, both through taming the neuroinflammation and boosting adaptive immunity.

RSV has also been analyzed in terms of its possible anti-tumor properties. Cancer initiation and progression are caused by genetic and/or epigenetic alterations. Acetylation of histone and non-histone proteins plays a crucial role in the epigenetic regulation of gene expression. Modification status of histones is controlled by the balance between activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Imbalance between the activity of the enzymes mentioned above is associated with various forms of cancer. Natural plant-derived compounds have been reported to show potential HDAC-inhibitory activity. There are several HDAC inhibitors of natural and dietary origin that include not only RSV, but also butein, protocatechuic aldehyde, kaempferol (occurring in grapes, green tea, tomatoes, potatoes and onions), sinapinic acid (present in wine and vinegar), diallyl disulfide (present in garlic) and zerumbone (present in ginger) [23]. While the deacylated status of histones promotes genomic stability and prevents transcriptional noise (which takes place in healthy young cells), general hyperacylation of histones can impair genomic stability and accelerate telomere attrition, which can be a desirable effect within tumor cells. Moreover, acylated histones can make DNA more accessible to some transcription factors, which can facilitate the synthesis of some *anti*-oncoproteins. This is why the inhibition of HDAC can provide some anti-tumor efficacy. Furthermore, RSV can activate the class III HDAC family (sirtuins, especially SIRT1), while inhibiting other classes of HDAC [24]. Details concerning various clinical trials assessing RSV, including its potential anti-tumor activity, have been reviewed by Berman et al. [25].

RSV also plays a significant role in the prevention of obesity and related metabolic disorders. Research studies reported that RSV mimics

the effects of calorie restriction via activation of SIRT1, which is closely connected to a cellular energy levels and energy homeostasis. Rodent studies confirmed beneficial effects of RSV supplementation on mitochondrial function, glucose metabolism, liver fat accumulation and body composition [26,27]. RSV can prevent obesity through activating SIRT1-dependent metabolic pathways in hepatocytes (fatty acid oxidation and gluconeogenesis) and in POMC-releasing neurons of the hypothalamus, exerting a direct inhibitory effect towards feeding [28–33].

Another area where RSV can be used is prevention of age-related diseases. RSV and pterostilbene have been reported to have some anti-aging properties, through modulating the hallmarks of aging, such as oxidative damage, inflammation, telomere attrition and cell senescence [34,35]. Anti-aging effects of RSV are thought to depend on SIRT1 activation, although the activation itself has once been questioned. However, most recent studies confirmed that RSV does activate SIRT1 *in vivo*, through a process called assisted activation (i.e. activation in the presence of natural substrates) [36–38]. SIRT1, in turn, can exert some anti-aging effects through histone deacylation [39–42], neuroprotection [43–49] and direct interactions with some transcription factors [50–52].

RSV exerts some protective actions against atherosclerosis-associated endothelial dysfunction. According to some authors, antiatherogenic effects of RSV comprise mainly activation of SIRT1, adiponectin and calprotectin (S100A8/A9) [53–55].

In addition, *in vitro* studies [56] have shown inhibitory action of RSV towards platelet aggregation. *Cis*-RSV at the concentration of $1 \times 10^{(-5)}$ and $1 \times 10^{(-6)}$ M has been able to decrease collagen-induced platelet aggregation by 43.5 (± 11.4%) and 26.8 (± 14.6%), while *trans*-RSV has shown slightly weaker effect at the same concentrations. The research has been done using platelet-rich plasma collected from healthy volunteers. *In vivo* assessment of RSV actions cardiovascular diseases, obesity, diabetes and other age-related diseases in humans and laboratory animals has been reviewed by Kulashekar et al. [57]. This study, like many others, confirmed the RSV safety and tolerability but, at the same time, relatively poor bioavailability when applied in currently available preparations.

Despite incomplete understanding of the mechanisms underlying RSV action on glucose metabolism in adipocytes, some *in vitro* studies have shown that RSV treatment substantially increases glucose uptake in insulin-resistant 3T3-L1 adipocytes [58,59]. In another study, rats have been divided into two groups, the first group consisting of aged and fat animals, while the second including only exercised rats, additionally treated with RSV. In the rats from the first group, RBP4 mRNA and protein expression in visceral adipose tissue, as well as glucose and RBP4 concentration in the plasma were higher, while insulin sensitivity was lower than in the rats from the second group [60].

Another possible use of RSV is interval or continuous therapy, alleviating the abnormalities associated with non-alcoholic fatty liver disease (e.g. chronic inflammatory response, excessive lipogenesis, imbalance between FFA synthesis and degradation) [61,62].

Summing up, RSV is regarded as possibly protective against AD mainly because of its antioxidative, anti-inflammatory, and neuroprotective actions (some of them being presumably dependent on the assisted activation of SIRT1).

2.4. Pathomechanisms of Alzheimer's disease - what is already known?

AD - a neurodegenerative disease - is the most frequent cause of dementia in the world. It is estimated that approximately 5.4 million of America's population suffer from AD, and this number may increase to 13.8 million by the middle of the 21st century, mainly due to the aging phenomenon [63]. There are two main forms of the disease - familial and sporadic. Most patients suffer from the sporadic form, which is characterized by late onset (approximately 80–90 years of age) and failure of the amyloid-β peptide (Aβ) clearance from the brain, as a main component of its pathomechanism [64].

No particular gene responsible for the onset of this medical condition has been found. It is unknown why some people develop the disease, whereas others with the same set of genes do not. However, the best known genetic risk factor responsible for the excessive immune response and inflammation in the late-onset AD form, is possessing APOE ε4 allele. Recently, more genes playing a major role in the pathogenesis of AD have been discovered, including *HLA-DRB5/DRB1, INPP5D, MEF2C, CR1, CLU and TREM2* [65].

The familial form of AD affects about 10–25% of all patients, who usually have ≥2 relatives with a history of AD in their families. The onset is late (60–65 years) in approximately 95% of cases and early (age < 65 years) in the remaining 5%. Three main genes involved in the early-onset AD form include: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) [66].

AD pathomechanism consists of the abnormal accumulation of Aβ aggregates, occurring when, instead of being metabolized by α-secretase, APP is abnormally split by β- and γ-secretases, which leads to the excessive extracellular accumulation of Aβ aggregates in the cortex and hippocampus of the affected brain. In addition, hyperphosphorylation can be observed in microtubule-associated neuronal tau protein which normally takes part in regulation of brain homeostasis [67]. Those changes affect mainly medial temporal lobes and associative neocortical structures [68].

Abnormal deposition of Aβ aggregates can interfere with neuronal function and initialize the neuroinflammatory response related to the excessive activation of microglia, astrocytes and macrophages. It is worth emphasizing that the inflammatory response is driven mainly by hyperactivated microglia [69]. Excessive activation of these cells has been reported both in AD patients and in animal models; it is associated with increased levels of specific chemokines, cytokines, free radicals and NO.

Neurons contain a large number of mitochondria, allowing them to metabolize oxygen intensively, which can lead to production of great amounts of ROS by several enzymes, including NO synthase (iNOS) and cyclooxygenase-2 (COX-2). ROS are produced in dysfunctional mitochondria during oxidative stress, which can result in damage to cellular proteins and nucleic acids. This process can lead to lipid peroxidation and loss of phospholipid membrane integrity. In addition, neurons may be exposed to superoxide moieties produced by the adjacent microglial cells [70].

ROS increase the Aβ production, which can in turn enhance the oxidative stress. This basically explains a positive feedback loop between ROS production and Aβ accumulation – a vicious circle that may accelerate the progression of AD. Aβ aggregates can activate astrocytes and microglia, promoting the release of large amounts of pro-inflammatory mediators that in turn increase the production of insoluble Aβ aggregates. Therefore, antioxidants, including RSV and other polyphenols, have been shown to protect CNS from Aβ-induced neurotoxicity, through reducing iNOS activity and thus alleviating lipid peroxidation in neurons. In addition, they can upregulate heme oxygenase-1 (HO-1), thus further reducing the effects of the oxidative damage.

The fact that some non-steroidal anti-inflammatory drugs (NSAIDs) protect against AD may also support the neuroinflammatory pathogenesis of this neurodegenerative disease. There is vast evidence that the use of NSAIDs is inversely correlated with AD incidence (relative risk - RR, 0.72; 95%CI, 0.62–0.84) and the negative correlation is even stronger for patients using NSAIDs in a long-term perspective (RR, 0.36; 95%CI, 0.17–0.74). Even with aspirin, the risk of AD is lower in its users (RR, 0.77; 95%CI, 0.63–0.95) compared to non-users [71].

Tau protein, a soluble microtubule-associated phosphoprotein (MAP), which occurs in both central and peripheral nervous systems, plays a crucial role in stabilizing the microtubules. Phosphorylation of tau protein is regulated by many kinases and the pathological aggregation of tau protein or glial fibrillary tangles in the human brain leads to the production of a hyperphosphorylated, insoluble form of

tau. Such abnormal conditions, known as tauopathies, include over twenty diseases, including AD, progressive supranuclear palsy (PSP), Pick's disease, corticobasal degeneration or post-encephalitic Parkinsonism.

Tau protein polymerization within neurofibrillary tangles results in the loss of its tubulin binding and microtubule confluence-supporting function. Therefore, inhibition of abnormal hyperphosphorylation of tau proteins can be another therapeutic strategy for AD, as well as other tauopathies.

2.5. How RSV can counteract the pathomechanisms discussed above?

2.5.1. Antioxidative actions of RSV

Neuroprotective effects of RSV are primarily attributed to its antioxidative action. As it has already been mentioned, the high metabolic demand of neurons gives them three typical traits: a large number of mitochondria, an intense oxygen metabolism and fairly large production of ROS [72]. Due to the presence of the –OH group, RSV can neutralize ROS, as well as reactive forms of nitrogen.

In addition, neurodegenerative diseases, such as AD, reduce the capacity of antioxidant systems, resulting in the elevated levels of both oxidative stress markers and markers of protein, lipid and DNA damage. Polyphenols increase the expression of antioxidant enzymes such as superoxide dismutase, catalase or glutathione peroxidase [73], which can in turn reduce the activity of ROS-generating enzymes, such as xanthine oxidase, COX-1, and NADPH-oxidase [74].

RSV can reduce glutathione and inhibit COX-2, thus preventing both the action of free radicals and production of pro-inflammatory eicosanoids. In addition, RSV can decrease the activity of TNF-alpha and reduce the secretion of IL-1, which may also account for its anti-inflammatory properties. Serum level of C-reactive protein (CRP) is also less likely to be elevated in humans regularly consuming dietary sources of RSV [75]. Turner et al. [76] performed a randomized, double-blind, placebo-controlled trial of RSV for AD.

However, the conclusion from the present study suggests has suggested that further studies may be required to interpret AD biomarker changes during RSV treatment.

2.5.2. Counteracting peptide aggregate deposition and assisted activation of SIRT1

According to *in vitro* research findings, polyphenols can directly prevent the aggregation of peptides and proteins - so typical for AD [77]. RSV is able to destabilize already existing amyloid aggregates and transform them into non-toxic products [78,79].

Jia et al. [80] report that RSV can inhibit Aβ production and aggregation, while stimulating its clearance through a number of possible mechanisms, which provides a rationale for the use of RSV in AD treatment.

RSV activates NAD-dependent histone deacetylases – sirtuins [81,82], which are called “longevity enzymes” because of their properties [83–85]. SIRT1 especially can exert neuroprotective actions through the inhibition of Aβ-aggregates formation by upregulating alpha-secretase – the enzyme which is “competitive” to β-secretase [86]. In addition, sirtuins inhibit inflammatory response and increase neuroplasticity, which can also be useful in AD through preventing the development of its three typical features: loss of dendritic spikes, decrease in synaptic plasticity, and chronic inflammation [87].

2.5.3. Tau protein demethylation

RSV promotes tau protein demethylation at lysine residues, which allows its polyubiquitination and proteasomal degradation. In addition, it activates phosphatase 2 A (PP2A) protein which is the most important phosphatase capable of tau protein dephosphorylation. Effective degradation of tau protein prevents formation of neurofibrillary tangles [88]. Fig. 3 illustrates main directions and mechanisms of RSV neuroprotective action.

2.5.4. Evidence for RSV-dependent neuroprotection from in vivo and in vitro studies

Recently, the effects of RSV on the brain status of control non-transgenic (NoTg) and AD transgenic (3xTg-AD) mice fed with RSV-supplemented diet (100 mg/kg) have been studied to find the mechanisms underlying resistance to age-related AD. The results show that 10-month RSV supplementation not only robustly protects from memory loss and brain pathology in 3xTg-AD mice, but also provides cognitive improvement in healthy NoTg mice. Furthermore, RSV supplementation reduces anxiety and accumulation of Aβ and p-tau aggregates in the hippocampus of the 3xTg-AD mice. The findings show increased levels of amyloid-degrading enzyme, neprilysin, reduced activity of amyloidogenic secretase BACE1, as well as an increased level of proteasome protein and enhancement of proteasome activity - in both NoTg and 3xTg-AD mice. RSV also activates 5′AMP-activated protein kinase (AMPK), subsequently upregulating SIRT1, resulting in activation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and cAMP response element-binding protein (CREB) in both types of mice, which leads to consecutive beneficial effects. The results suggest that RSV may enhance cognitive capacity, as well as provide neuroprotection against Aβ and tau protein-related pathologies. In both mouse strains, the amendment of proteostasis by RSV has been observed, which suggests that proteasomal degradation of abnormal proteins is a crucial preventive mechanism against diseases related to their accumulation, with subsequent neurodegeneration [89].

Other authors have studied the inhibitory effects of RSV towards inflammatory response in rat astrocytes and N9 microglia cell lines, by observing the changes in the expression of pro-inflammatory factors, chemokines, as well as cell cycle markers and adhesion molecules on the cell surface [90]. In astrocytes, both proliferation index and the levels of TNF-α, IL-1β and MCP-1 levels in the supernatant have been reduced by using 5, 12.5, and 25 μM RSV. Likewise, in N9 microglia, the levels of IL-1β, IL-6 and NO in the supernatant, as well as CD40 and MHC-2 expression have been significantly decreased by the treatment with 10, 20 and 40 μM RSV solutions. Furthermore, immunofluorescent findings have shown that RSV can diminish nuclear translocation of NF-κB/p65 and increase the expression of NF-κB/p65, but at the same time, it can reduce the expression of p-IκB in the cytoplasm in both astrocytes and N9 microglia. Summing up, the results show that RSV reduces inflammation in rat astrocytes and N9 microglia, so the NF-κB signalling pathway can be a significant target in the treatment of AD.

In another study [91], using Senescence-accelerated mouse-prone 8 (*SAMP8*) age-related AD mouse model, possible beneficial effects of RSV towards cognitive performance have been evaluated. It has been found that a long-term dietary supplementation with RSV (150 mg/kg a day) significantly improves cognitive skills of the *SAMP8* mice in Morris water maze tests. Furthermore, the treatment leads to a significant improvement in various AD markers in *SAMP8* brains (reduced level of Aβ-peptide 42, lower degree of phosphorylation of the microtubule-associated protein tau, increased phosphorylation of glycogen synthase kinase 3β (GSK-3β) at Ser9, and decreased expression of TNFα, IL-6, and IL-1β). RSV can also markedly reduce the level of ROS in serum. These results show that an RSV-supplemented diet can effectively prevent age-related cognitive deficits and neurobiochemical alterations, as well as contribute to AD prevention.

In the study by Mohan et al. [92], the authors describe neuroprotective effect of RSV in AD, related to the depolymerisation of Aβ fibrils. Although the molecular targets of RSV remain unknown, it has been shown that RSV reduces Aβ levels by promoting its proteasomal degradation. As it has been mentioned before, Aβ peptides can activate microglial cells through several Toll-like receptors (TLRs) including TLR4. RSV affects IκB Kinase (IKK) and IκB phosphorylation, which prevents activation of STAT1, STAT3 and NF-κB, and thus prevents the pro-inflammatory effect of Aβ towards macrophages. Finally, in mouse models of cerebral amyloid deposition, RSV treatment significantly reduces microglial activation related to the amyloid deposition.

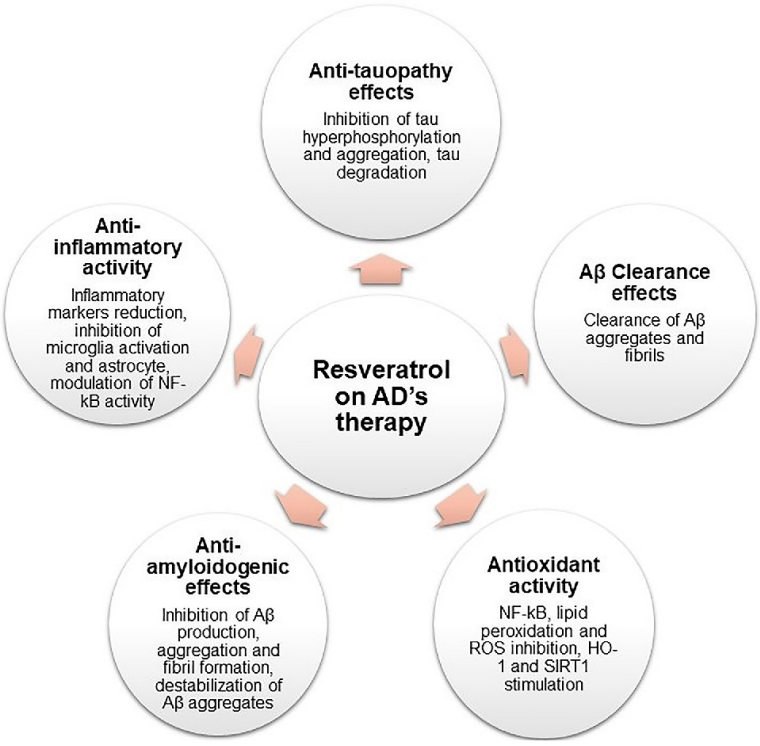

Fig. 3. Resveratrol mechanisms of action in Alzheimer's disease prevention and therapy.

RSV activates SIRT1 - an NAD + -dependent deacetylase that regulates diverse genes and proteins involved in cell proliferation, apoptosis, senescence, and differentiation. SIRT1 activation by RSV protects against oxidative stress, metabolic decline, inflammation as an element of AD pathogenesis, as well as improves synaptic plasticity, learning and memory. It has been reported that reduced SIRT1 activity in neurons is an early prognostic marker of AD. In the study by Wang et al. [93], using a mouse model of AD, the researchers focused on combined effects of applying human Umbilical Cord Mesenchymal Stem Cells Subpopulations (hUC-MSCs) and RSV as an effective approach to AD treatment via SIRT1 signalling. Combining hUC-MSCs transplantation with RSV treatment results in better neuroprotection of mice affected by AD than any individual proceeding alone, which may be linked to SIRT1 signalling. RSV promotes hUC-MSCs engraftment and neural repair in a mouse model of AD.

In another interesting *in vitro* research [94], the authors tried to find whether RSV can significantly reduce Aβ-induced oxidative damage in PC12 cells and to investigate whether the protective effect of RSV is associated with mitophagy. Mitophagy, as a form of autophagy responsible for mitochondrial quality control, is the selective degradation and intracellular recycling of damaged mitochondria. It has been demonstrated that mitochondrial damage can lead to decreased mitochondrial membrane potential, resulting in mitophagy. In addition, mitochondrial dysfunction can contribute to oxidative stress, which is regarded as one of the causative agents in AD and other neurological diseases. Thus, selective removal of dysfunctional mitochondria through mitophagy can be an effective way to limit the neuronal oxidative damage.

Substantial evidence suggests that RSV-induced mitophagy plays a protective role against oxidative damage in the *in vitro* model of AD. This research can help in understanding the biological mechanism of mitophagy activation by RSV.

APP is cleaved by β- and γ-secretases, which altogether promotes the production of Aβ-aggregates. RSV promotes intracellular clearance of Aβ without affecting its production, through activating AMPK independently of SIRT1. The phosphorylation of PKC is induced by RSV and subsequently plays a major role in neuroprotection. RSV prevents the formation of Aβ-Fe, Aβ-Cu, and Aβ-Zn complexes and thus attenuates their toxicity.

ROS are produced in damaged mitochondria during oxidative stress and play an important role in apoptosis. RSV inhibits iNOS and COX-2 activity, while upregulating heme oxygenase 1 (HO-1) to attenuate oxidative damage. RSV inhibits the expression of the ROS-producing enzyme Nox4, while at the same time promotes the expression of ROS-inactivating enzymes - SOD1 and GPx1.

Aβ aggregation is responsible for the activation of astrocytes and microglia, which release cytokines, such as IL-1β, IL-6, and TNF-α, all of them being transcriptionally controlled by NF-κB. RSV can inhibit PGE_2 production in activated microglial cells. Finally RSV inhibits STAT1, STAT3 and IκBα phosphorylation, as well as NF-κB binding to DNA through SIRT1-dependent pathways. All these findings can explain at least some of the mechanisms of neuroprotective actions of RSV – direct or indirect.

2.5.5. Summary of evidence

According to numerous research studies, RSV may prevent AD or delay its onset, but there are very few studies pertaining to humans. To confirm RSV neuroprotective effects in reference to humans, more studies on humans should be performed. The studies may include prospective clinical trials in individuals with a familial risk of early-

Fig. 4. Resveratrol delivery systems.

onset AD, as well as in individuals suffering from AD, at its early stages.

2.5.6. Limitations

There are few research studies pertaining to humans. RSV penetration through BBB is not complete. There are numerous studies aimed at improvement of RSV oral bioavailability, and hence - its effect in CNS. These strategies include RSV nanoencapsulation in various structures, such as liposomes or micelles, as well as modifications of RSV structure, including methoxylation and hydroxylation of the RSV aromatic rings [95]. In addition, some novel drug delivery systems have been tested to increase beneficial effects of RSV [96]. Methods that have been used to improve RSV bioavailability are illustrated in Fig. 4.

Both, RSV dosage and the optimal age at which the prevention should be started, are yet to be thoroughly settled.

3. Conclusions

As it has been already written above, neurodegenerative diseases are constantly on the rise. They usually have a severe course, leading to the impaired functioning of the organism. The inflammatory response within the CNS plays a significant role in the pathogenesis of neurodegenerative diseases, including AD. Numerous studies mentioned above suggest that RSV may exhibit some neuroprotective effects, related both to its antioxidant properties and to its inhibitory capabilities towards formation of protein aggregates and neurodegeneration-associated peptides (Aβ, alpha-synuclein), thus reducing their cytotoxicity. In addition, RSV can activate proteins essential for proper neuron functioning (e.g. sirtuins), and prevent the development of chronic inflammation, through downregulation of some pro-inflammatory cytokines. Numerous studies, including one clinical trial, have investigated possibly neuroprotective effects of RSV, although two main limitations that have been observed, included RSV poor solubility in water, and hence, its limited bioavailability [97]. Nevertheless, RSV mechanisms of action indicate that it could be used in AD prevention, especially in reference to some familial forms of AD, if only its bioavailability were significantly improved, using some methods illustrated in Fig. 4. Therefore, successful use of RSV for AD prevention can depend mainly on the effective improvement of its bioavailability.

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The author contribution

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Data Collection: Justyna Komorowska, Mateusz Wątroba, Dariusz Szukiewicz.

Statistical Analysis: Justyna Komorowska.

Data Interpretation: Justyna Komorowska, Mateusz Wątroba, Dariusz Szukiewicz.

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Declaration of competing interest

The authors declare no conflict of interests.

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The Role of Glucose Concentration and Resveratrol in Modulating Neuroinflammatory Cytokines: Insights from an In Vitro Blood–Brain Barrier Model

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Background: The prevalence of type 2 diabetes mellitus is rising, presumably because of a coexisting pandemic of obesity. Since diabetic neuropathy and neuroinflammation are frequent and significant complications of both prolonged hyperglycemia and iatrogenic hypoglycemia, the effect of glucose concentration and resveratrol (RSV) supplementation on cytokine profile was assessed in an in vitro model of the blood–brain barrier (BBB).

Material/Methods: The in vitro model of BBB was formed of endothelial cells and astrocytes, which represented the microvascular and brain compartments (MC and BC, respectively). The BC concentrations of selected cytokines – IL-10, IL-12, IL-17A, TNF-α, IFN-γ, GM-CSF in response to different glucose concentrations in the MC were studied. The influence of LPS in the BC and RSV in the MC on the cytokine profile in the BC was examined.

Results: Low glucose concentration (40 mg/dL) in the MC resulted in increased concentration of all the cytokines in the BC except TNF-α, compared to normoglycemia-imitating conditions (90 mg/dL) ($P<0.05$). High glucose concentration (450 mg/dL) in the MC elevated the concentration of all the cytokines in the BC ($P<0.05$). RSV decreased the level of all cytokines in the BC after 24 h following its administration for all glucose concentrations in the MC ($P<0.02$). The greatest decline was observed in normoglycemic conditions ($P<0.05$).

Conclusions: Both hypo- and hyperglycemia-simulating conditions impair the cytokine profile in BC, while RSV can normalize it, despite relatively poor penetration through the BBB. RSV exhibits anti-neuroinflammatory effects, especially in the group with normoglycemia-simulating conditions.

Keywords: Astrocytes • Blood–Brain Barrier • CSF2 Protein, Human • Cytokines • Endothelial Cells • Hyperglycemia • Hypoglycemia • IL12A Protein, Human • Interferon gamma (1-39) • Resveratrol • Tumor Necrosis Factor alpha (36-68)

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Background

The prevalence of diabetes mellitus (DM) and impaired glucose tolerance (IGT) is rising worldwide [1]. Both of these conditions are correlated with an increased incidence of innate immunity dependent low-grade systemic inflammation [2-4]. The presence of such an inflammatory response within the blood–brain barrier can be potentially harmful, disrupting its integrity and increasing permeability, which may in turn promote penetration of potentially toxic or immunogenic substances to the central nervous system [5]. Furthermore, hyperglycemia can promote mitochondrial oxidative stress through enhancing glucose influx to insulin-independent cells [6,7], as well as biosynthesis of advanced glycation end-products (AGE) that may exert pro-inflammatory effects through activating receptors for advanced glycation end-products (RAGE) and some pattern-recognizing receptors (PRR), thus promoting neuroinflammation [8,9]. Therefore, both DM and IGT increase the risk of neuroinflammatory response through excessive stimulation of innate immunity as a result of both increased ROS production and AGE biosynthesis. Increased ROS and AGE concentrations can promote release of pro-inflammatory cytokines through direct or indirect activation of NF-κB signaling pathways [9]. Altered glucose concentration in the plasma can induce an adaptive response within the BBB, and thus change the rate and characteristics of glucose transport from the plasma to the brain parenchyma. Moreover, hyperglycemia-dependent oxidative stress can activate an innate immunity hyperglycemia-dependent inflammatory response within the BBB, which can disrupt its integrity and increase permeability [5].

In the course of hyperglycemia, an excessive influx of glucose to insulin-independent cells results in accelerated rates of oxidative glycolysis and tricarboxylic acid cycle (TCA). Because TCA reactions are coupled with transforming NAD+ into NADH, while neither NAD+ nor NADH can cross mitochondrial membranes through simple diffusion, the increased rate of TCA can decrease the NAD+/NADH ratio in the mitochondria. At the same time, intracellular ATP concentration is high, which can inhibit electron transport chain (ETC), a normal acceptor of free electrons carried by NADH. This can produce an increased risk of non-enzymatic electron drop into molecular oxygen (ie, ROS production), with subsequent NF-κB activation by ROS [10,11].

Since increased ROS concentrations can inhibit GADPH, oxidative stress can redirect glucose metabolism from oxidative glycolysis to the AGE biosynthesis pathway, among others [6,7]. In turn, increased AGE concentrations can exert pro-inflammatory effects, both through RAGE activation and through activation of some pattern-recognizing receptors (eg, TLR4), which finally stimulates NF-κB-dependent signaling and release of some pro-inflammatory cytokines. Therefore, pro-oxidative effects of hyperglycemia in endothelial cells and neurons can promote neuroinflammation [9]. In these conditions, the role of astrocytes becomes particularly important—firstly, because astrocytes are an essential part of the BBB in modulating and maintaining the barrier properties of the brain endothelial cells [12]; and secondly, because astrocytes cooperate with neurons on several levels, including neurotransmitter trafficking and recycling, ion homeostasis, energy metabolism, and defense against oxidative stress [13,14].

Increased ROS concentration in the mitochondria produces a risk of mitochondrial DNA (mtDNA) damage, so it also activates mechanisms which protect the cell from the effects of such damage (eg, HSP-60 expression). However, some HSP-60 molecules may be transported to the cell membrane, where they can activate extracellular innate immunity mechanisms (eg, pattern-recognizing receptors [PRRs] on leukocytes) [15].

Although glycogen synthase is allosterically activated in CNS by glucose-6-phosphate (G6P), and neurons also have an active glycogen metabolism, brain glycogen is stored in astrocytes but not in neurons [16,17]. Thus, neurons require continuous support of glucose from the circulatory system [18]. Glucose depletion in neurons can result in a secondary ATP depletion, with hypoglycemic coma, or even cell necrosis, as possible results. Repeated hypoglycemic episodes, including an adverse effect of treatment with blood-sugar-lowering medication in diabetes, is associated with long-term cognitive deficits [19]. Hypoglycemia has been also linked to increased concentration of pro-inflammatory cytokines, with unclear mechanism of this linkage [20,21]. In patients suffering from type 2 diabetes mellitus (T2DM), induction of hypoglycemia has been related to significantly elevated concentrations of both oxidative stress and inflammatory response markers – both in blood and in urine – after 24 h from the onset of hypoglycemia, whereas in healthy individuals, such a correlation has not been reported [22].

Summing up, long-term hyperglycemia can promote neuroinflammation, both through its pro-oxidative effects with subsequent activation of NF-κB signaling by ROS, and through its AGE biosynthesis-promoting effects, with subsequent activation of RAGE and some PRR receptors (eg, TLR4) [6,7,23-25]. The effects of prolonged hyperglycemia within the CNS are graphically presented in **Figure 1**.

Neuroinflammation can increase the risk of neurodegenerative diseases such as Alzheimer disease and Parkinson disease, stroke, and multiple sclerosis because it changes the phenotype of microglial cells from quiescent, homeostatic to pro-inflammatory, which results in increased activity of inflammasomes, such as the NLRP3 inflammasome. This in turn stimulates release of some pro-inflammatory cytokines, such as IL-1β, and impairs microglia-dependent Aβ clearance from extracellular spaces [26-33].

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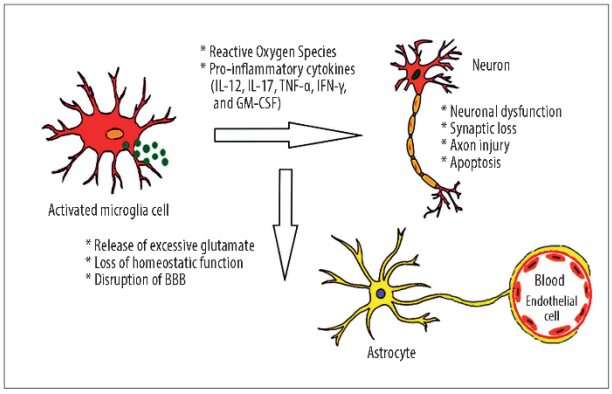

Figure 1. Effects of hyperglycemia within central nervous system. *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

Resveratrol (RSV) is a naturally occurring polyphenolic compound, endowed with some anti-oxidative, immunomodulating, anti-neuroinflammatory, and anti-tumor properties [34-38]. Because the pro-neuroinflammatory tendency depends on the nutritional or metabolic status of the body, it is worth studying whether this status, expressed by plasma glucose concentration, can modulate anti-neuroinflammatory properties of RSV.

Therefore, the aim of the study was to assess the effect of different glucose concentrations on the cytokine profile within an in vitro model of BBB, as well as to evaluate anti-neuroinflammatory effects of RSV in hypo-, normo-, and hyperglycemia-mimicking conditions.

Material and Methods

Ethics Statement and General Data

According to the latest version of the Declaration of Helsinki, since the research involves the commercially available HCMEC/D3 cell line, it does not have any clinical implications and does not involve human subjects directly, it may not be necessary to obtain additional consent. Nonetheless, this study has been reported to and accepted by the Bioethics Committee of the Medical University of Warsaw on 14 December 2020 (no. AKBE220/2020) as not requiring specific approval from the Committee, given the exclusive use of commercially available human cell lines.

The experiment was conducted from January to May 2021 in the Warsaw Medical University Center of Preclinical Research and Technology (CePT) in an in vitro model of the BBB, consisting of human astrocytic cells and endothelial cell lines with a separating membrane containing 0.4-µm-wide pores. This was an experimental and interventional study aimed at evaluation of the effect of glucose concentration in the MC (which simulates intravascular fluid in the study conditions) on the pro-inflammatory cytokine profile, as well as on the anti-inflammatory action of RSV in the BC (which simulates cerebrospinal fluid in the study conditions).

In Vitro Model of BBB

In this study, a 2-component in vitro model of the BBB, consisting of endothelial cells and astrocytes, was used. The HCMEC/D3 human endothelial cell line, containing cells obtained from cerebrovascular endothelium, was purchased from Cedarlane Cellutions Biosystems (Burlington, ON, Canada; catalog # CLU512), prepared from cerebral capillary endothelial cells by transduction with lentiviral vectors carrying the SV40 T antigen and human telomerase reverse transcriptase. This cell line is commercially available (Sigma-Aldrich; cat. no. SCC066), standardized, and, according to the producer, it can be used in cellular and molecular studies of the central nervous system or as a single-cell model of the human BBB [39]. To provide proliferation of endothelial cells with 100% confluence, dedicated EBM-2 medium was used, containing 10% fetal bovine serum, 1x chemically defined lipid concentrate, 5.7 mM ascorbic acid, 0.0125 mM bFGF- human basic fibroblast growth factor, 1M HEPES, 2.8 mM hydrocortisone, penicillin, and streptomycin (Merck KGaA, Darmstadt, Germany; cat. #C-22211). Human astrocytes were derived from human brain tissue (Thermo Fisher Scientific-Gibco™; Waltham, MA, USA; cat. # N7805100). According to the producer, this cell line can be used for studying human neurological pathways and diseases. To provide proliferation of astrocytes with 100% confluence, basal DMEM medium for astrocyte culture was used, containing: 10% One Shot Fetal Bovine Serum, 1x N-2 Supplement (all purchased from Thermo Fisher Scientific-Gibco™, cat. # A1261301), and penicillin and streptomycin (Merck KGaA, Darmstadt, Germany; cat. #C-22211). Astrocyte culture was performed according to the protocol of the supplier of the astrocyte line and the manufacturer of the medium

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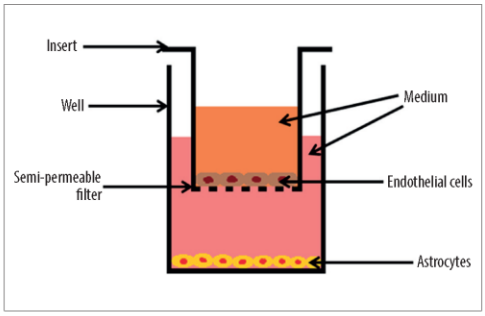

Figure 2. The cross-section scheme of in vitro model of blood–brain barrier containing co-culture of endothelial cells and astrocytes. The basement membrane with pore diameter 0.4 µm. *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

(https://assets.fishersci.com/TFS-Assets/LSG/manuals/Gibco_HumanAstrocytes_and_medium_man.pdf).

Both cell cultures were held in 5 special culture bottles obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland), the mean culture surface was 75 square centimeters for endothelial cells and 25 square centimeters for astrocytes. The culture growth duration to obtain 100% confluence was 3 and 4 days. For measurements of the confluences of the cultured adherent cell lines, both the HCMEC/D3 and the human astrocytes, non-invasive, non-destructive, and label-free method were applied [40]. In this method, which eliminates sample manipulation, images of the cultured cells are captured by photography under a routine inverted phase-contrast microscope with LED (Leica DMIL LED; Leica Microsystems CMS, Germany) using a digital camera (Nikon D3100; Nikon Imaging Japan, Inc., Japan) with a dedicated camera lens adaptor (Proscope, Bodelin Technologies, Inc., Oregon, USA). Next, the images were analyzed for confluence using ImageJ 2.1.4.7 i1 freeware, and the measure of confluence was expressed as an area fraction (AF).

After 100% confluence was reached within both types of cells, the number of living cells was counted. It was mixed with trypan blue dye, and 10 mL of the mixture was placed in the chamber's slide, then put into a Countess Automated Cell Counter (Thermo Fisher Scientific-Invitrogen).

The cell counter indicated that the number of living endothelial cells was 2 million per 1 ml of medium and the number of living astrocytes was 1 million per 1 mL of medium. The cells were placed into separate wells of nine 24-well plates (ThermoFisher Scientific-Gibco; cat. # A15690601); therefore, each well contained 6×10^4 endothelial cells and 3×10^4 astrocytes placed on inserts with transparent polyester (PET) membrane with pore diameter 0.4 µm, pore density 2×10^6 cm^2, and the culture surface 33.6 mm^2 per single well (Greiner Bio-One GmbH-ThinCert™, Frickenhausen, Germany, cat. # 662641). RSV, with its molecular mass of 228 Da and lipid soluble properties, should potentially be able to cross the BBB [41].

The wells were coated with a reduced growth factor basement membrane extract used for attachment and maintenance of human cells. After coating each of the 216 wells with Gibco Geltrex Matrix, 3×10^4 astrocyte suspension was put in each well with an Eppendorf pipette, so that after 24 h the astrocytes were fixed to the bottom and the medium portion was exchanged from DMEM to DMEM without glucose. Subsequently, the inserts were coated with type I collagen derived from rat tails (Merck C7661; cat. # C7661) and put into the wells. As soon as the collagen gained a gel-like consistence, 6×10^4 endothelial cells were placed into the inserts. After another 24 h, the medium was exchanged to a new medium containing a definite concentration of glucose. Finally, the BBB model consisted of endothelial cells corresponding to MC and astrocytes corresponding to BC of the BBB. The co-culture of cells was maintained in a humidified incubator (37°C, 5% CO_2). The scheme of the BBB in vitro model is presented in **Figure 2**. In addition, the diagram in **Figure 3** shows when each respective cell type was plated, when medium was changed for glucose incubation using the 3 concentrations, and then the washout periods with respect to cytokine collection.

To evaluate the effect of different glucose concentrations in MC on cytokine profile in BC and to further evaluate the anti-inflammatory action of RSV on the same model in hypo-, normo-, and hyperglycemic environments, 3 solutions with different glucose concentrations were set up in MC, which corresponded to the study groups I-III (**Table 1**).

Cytokine Concentration Measurement

Numerous existing studies suggest that differences in glucose concentration can change cytokine profile through modifying the production of pro-inflammatory cytokines [42,43]. To verify those findings, the concentrations of 6 different cytokines – IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF – were assessed 24 h after the washout of glucose-containing medium in the BC (stage 1 of the study) using a Multi-Analyte ELISArray Kit (Qiagen-Q4Lab, Hilden, Germany; cat. #336161).

Cytokine absorbance, measured in absorbance units (Au), which relate to its concentration and transmittance [44], were measured with a Multi-Analyte ELISArray Kit, strictly following the instructions of the manufacturer (https://www.qiagen.com/se/resources/resourcedetail?id=1da64594-2ac6-4bbd-983e-828cc74d6927&lang=en). The multi-well ELISA microplates were already coated with a panel of target-specific antibodies

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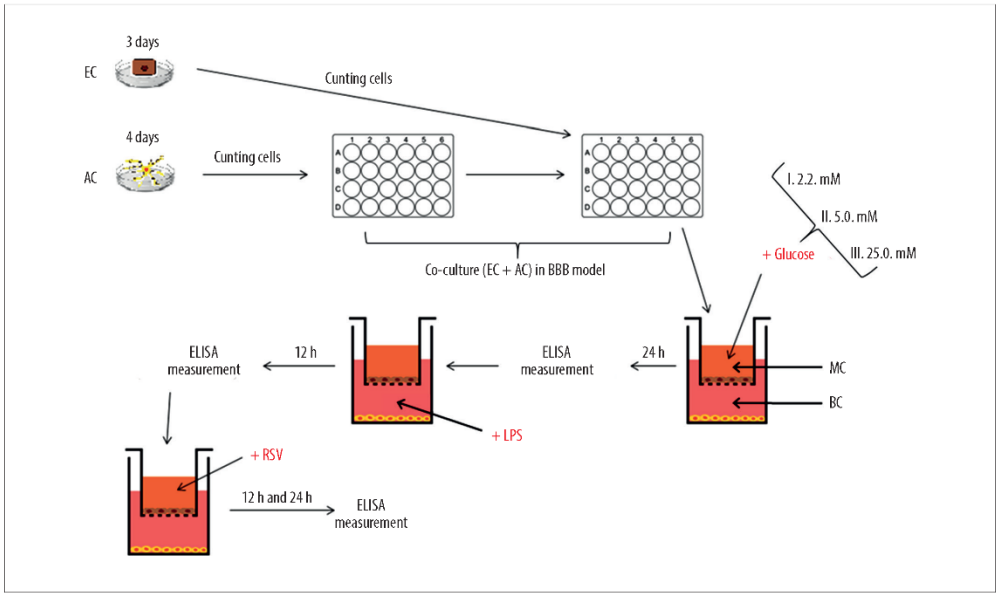

Figure 3. The diagram illustrating the process of establishing an in vitro blood–brain model and the stages of the study. EC – endothelial cells; AC – astrocytes; MC – microvascular compartment of BBB; BC – brain compartment of BBB; LPS – lipopolysaccharide' RSV – resveratrol. *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

Table 1. The 3 glucose concentrations used within microvascular compartment corresponding to decreased, normal, or increased blood glucose levels in vivo (hypo-, normo-, and hyperglycemia, respectively).

I. HYPOGLYCEMIA (N=72) – 40 mg/dL D-glucose (2.2 mM)
II. EUGLYCEMIA (N=72) – 90 mg/dL D-glucose (5.0 mM)
III. HYPERGLYCEMIA (N=72) – 450 mg/dL D-glucose (25 mM)

to capture different cytokines. Each ELISArray microplate included biological samples plus positive and negative controls (each given cytokine standard of known concentration and blank dilution buffer, respectively), to ensure that the experiment was performed properly. To set up a negative control, sample dilution buffer was added to each well in the first row, without an experimental sample. In the positive control, the final antigen standard cocktail was placed in each well in the last row. The samples representing study groups were added to the remaining rows in the ELISArray plate.

Concentrations of pro-inflammatory cytokines were recorded by means of Au, while the absorbance was measured at 450 nm of wavelength, and the readings at 570 nm were subsequently subtracted from the readings at 450 nm to eliminate the background dilution buffer effect. A monochromator based, PC-controlled microplate reader Biochrom Asys UVM 340 (Biochrom-Harvard Bioscience, Holliston, MA, USA) with a wavelength range 340-800 nm and the measurement range of 0-3.2 optical density (OD) was used. The reproducibility of this apparatus is defined by the manufacturer as 0.8% and 0.005 OD from 0.10 to 2.0 OD at 450 nm, whereas the accuracy was provided as 0.5% and 0.005 OD from 0.1 to 1.0 OD at 450 nm; and 1.0% and 0.010 OD from 1.0 to 2.0 OD at 450 nm. The measurements were performed 6 times, after which the average values were calculated in Au that corresponded to the given cytokine's concentration expressed in pg/ml ±standard error of measurement (SEM) as read from the standard curves (stage 1 of the study).

The Induction of Neuroinflammation

Lipopolysaccharide (LPS) solution was added into each well of the culture plate of BC to reach 0.2 µM and to induce the inflammatory response in BC. Concentrations of pro-inflammatory cytokines (IL-10, IL-12, IL-17A, TNF-α, IFN-γ, and GM-CSF) were counted by ELISA in all 3 study groups with different glucose concentrations after 12 and 36 h in BC. Therefore, 6 series of the measurements were carried out, with 6 measurements in each series (stage 2 of the study).

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Table 2. The set of controls established for experiments using the blood–brain barrier in vitro model.

Control groups	Basal medium	Glucose medium	LPS solution	RSV solution
1st stage of the study	+	–	–	–
2nd stage of the study	+	–	+	–
3rd stage of the study	+	–	+	+

The Influence of LPS on Astrocyte Morphology

We assessed the effect of LPS treatment on the morphology of astrocytic cells cultured in different glycemic conditions. Following the 36-h culture period after setting up the model of BBB for 3 study groups with or without LPS administration, the cells were fixed in 3% paraformaldehyde (PFA) in PBS for 30 min at room temperature, then they were paraffin embedded and stained with hematoxylin and eosin (H&E). Microscope images were acquired using an inverted cell culture Zeiss Primovert microscope equipped with light sources: HAL 35 W, 3W LED (Infinity Optics), and a Zeiss Axiocam 105 Colour camera. Images were analyzed using ZEN 2.3 software. The paraffin sections were examined under a light microscope (Leica DM 400B) by 2 independent experienced neuropathologists (50 images in each group), and photographs of the H&E-stained astrocytes were taken using a high-resolution camera attached to the microscope.

RSV's Penetration Through the BBB and Its Anti-Inflammatory Properties in BC

The next stage of the study was to estimate whether RSV applied to MC effectively penetrated the BBB (stage 3 of the study) and exhibited its anti-inflammatory properties through lowering the concentration of pro-inflammatory cytokines in BC (stage 4 of the study). RSV solution (final concentration 50 μM) was applied to the MC and measured in BC after 12 and 24 h in 3 study groups with different glucose concentrations. The RSV level in BC was estimated based on the value of standard concentration of RSV indicated in the instruction for the ELISA test and on the Au values. After the calibration curve was constructed, the exact concentration of RSV in samples was calculated from the equation of the graph.

Each set of measurements was conducted 6 times in each study group, with the average values presented in the next section.

Control Groups

To validate the research model (BBB) and to enable the correct interpretation of the results, the appropriate control groups were designed at the respective stages of the study (**Table 2**).

At the first stage of the study, the control group with a basal medium without any glucose concentration was set up. While performing the next stage of the study (LPS addition), the control group was established and consisted of a basal medium with 0.2 μM LPS but without any glucose solution.

At the last stage of the study, the control group with the basal medium containing 0.2 μM LPS as well as 50 μM RSV but no glucose medium was added. Each set of measurements was conducted 6 times in each control group.

During the development of this BBB in vitro model, the effect of culture media exchange on the integrity of the co-culture of endothelial cells and astrocytes was also evaluated. At that time, no disturbances in the integrity of the barrier with regard to the transfer rate of glucose were found. Due to the much smaller fluid volumes, there is no reason to believe that administration of RSV from the MC side and LPS from the BC side could affect the integrity of the BBB in a purely mechanical way. In turn, the assessment of changes in the functional integrity of the BBB resulted from the implementation of the purpose of this study.

Statistical Analysis

The statistical processing of the obtained data was performed using R programming language and its built-in functionalities (The R project for Statistical Computing; version 4.0.5) via the Mann-Whitney U test. All determinations were replicated 3 times and expressed as mean values with standard deviations.

A detailed description of the applied methods and the underlying principles is included in the subsequent *Results* sections. The results were considered statistically significant when *P* values were less then adjusted 0.05 ($P<0.05$).

Results

Cytokine Profile After 24 h Following Glucose Administration

Differences in glucose concentration in MC affected the pro-inflammatory cytokine profile in BC after 24 h following glucose

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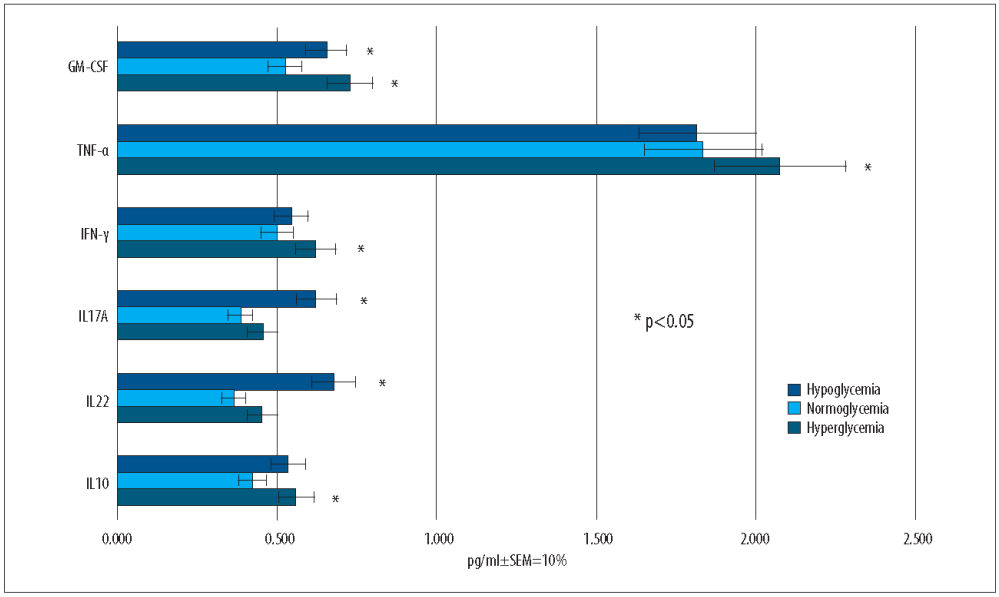

Figure 4. Mean values of cytokine concentrations (pg/ml) in brain compartment 24 h after different glucose concentrations applied in microvascular compartment. SEM – standard error of measurement (±10%). *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

addition. The mean concentrations of cytokines in reference to the corresponding glucose concentrations are presented in **Figure 4**.

To test whether there were statistically significant differences between the control and experimental groups (groups with basal medium vs groups with glucose medium), the Mann-Whitney U test was performed, which was also used to examine whether there was a difference in cytokine concentrations among the 3 experimental groups. The null hypothesis indicated that the mean of each of the cytokine levels was the same regardless of glycemia, with an alternative hypothesis that normoglycemia would have a lower mean level of cytokines.

The statistically significant results were marked with * in **Figure 4** when *P* values were less then adjusted 0.05 ($P<0.05$).

In 5 out of 6 cytokines (IL-10, IL-12, IL17A, INF-γ, and GM-CSF), the concentration was the lowest in the group containing normal glucose concentration (90 mg/dL), but not all of the results were statistically significant (statistically significant results are marked with * in **Figure 4**). The corresponding results in the group with abnormally low (40 mg/dL) and abnormally high (450 mg/dL) glucose concentrations differed in reference to individual cytokines. TNF-α concentrations were almost identical in groups imitating hypo- and normoglycemia, and the differences in concentrations were not statistically significant ($P>0.6$), while it was significantly higher ($P<0.05$) in hyperglycemia-imitating conditions (1.77±0.18 vs 2.063±0.2 [pg/ml ±SEM 10%]).

The level of IL-12 was highest in the samples obtained in the group imitating hypoglycemia and equaled on average 0.683±0.07 (pg/mL ±SEM 10%), whereas the lowest values were found in the samples from the normoglycemia-imitating environment (0.36±0.03 [pg/mL ±SEM 10%]), with *P* values <0.05. The mean level of IL-17A was the lowest in normoglycemic group, at 0.383±0.04 (pg/mL ±SEM 10%), slightly higher in the hyperglycemic group (0.435±0.04 [pg/mL ±SEM 10%]) and was significantly ($P<0.05$) higher in hypoglycemia-simulating conditions (0.6±0.06 [pg/mL ±SEM 10%]).

In the case of IL-10, the lowest level was obtained in the samples from the normoglycemic group (0.415±0.04 [pg/mL ±SEM 10%]), while in the other 2 groups its concentration was comparable and equaled on average 0.53 ±0.05 (pg/mL ±SEM 10%).

For INF-γ, the lowest values were found in the normoglycemic group (0.498 ±0.04 [pg/mL ±SEM 10%]), while higher in hypoglycemic (0.528±0.053 [pg/mL ±SEM 10%]) and hyperglycemic conditions (0.61±0.06 [pg/ml ±SEM 10%]), but the differences were significant only for the third group ($P<0.05$).

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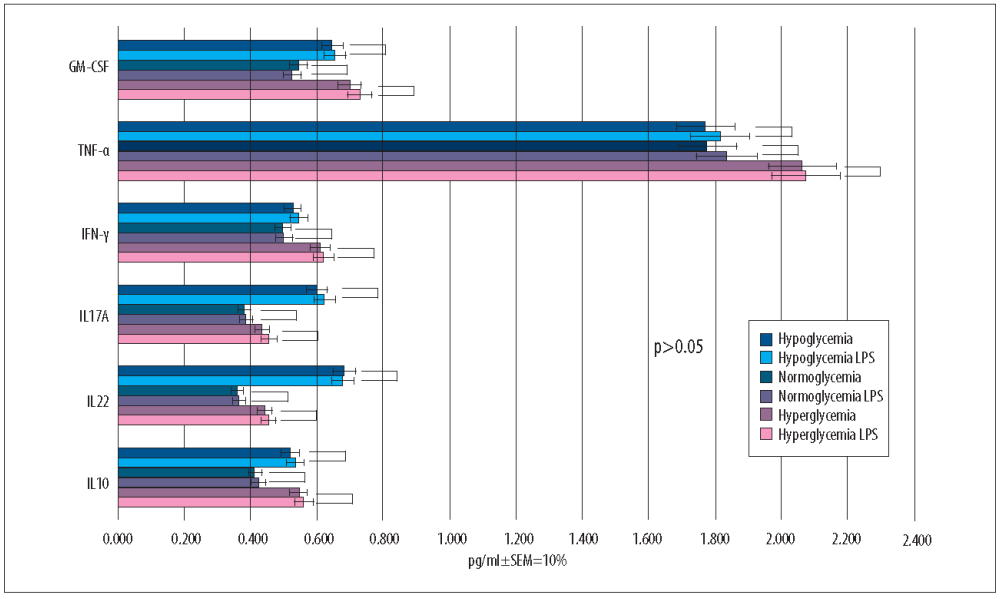

Figure 5. Mean values of cytokine concentrations (pg/ml) in brain compartment 12 h after LPS was been added to brain compartment in the 3 studied groups. In comparison, concentrations of the same cytokines in LPS-free conditions. SEM – standard error of measurement (±10%). *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

The mean level of GM-CSF was lowest in the normoglycemic group, and the value of 0.545±0.05 was significantly different ($P<0.05$) from the mean values obtained in the 2 other groups.

LPS Administration

At 12 h after administration of 650 µl of 1 µg/mL LPS solution to BC, the changes in concentrations for individual cytokines in BC were as shown in **Figure 5**.

At this stage of the experiment, the levels of 6 cytokines in BC as a reaction to adding LPS were compared. This test was performed separately for 12-h and 36-h periods and for each glucose level (normo-, hypo-, and hyper-glycemia).

The Mann-Whitney U test was performed based on the number of cytokines measured before LPS application. The null hypothesis was that the amount of each of the specific cytokines after 12 h and 36 h would be the same as before the application. The alternative hypothesis was that the amount of each of the specific cytokines would increase over time when LPS was applied.

The Mann-Whitney U test was used to assess whether there was a difference in cytokine concentrations between the 3 experimental groups following LPS administration. The obtained results presented in **Figure 5** showed there were no statistically significant differences in any experimental group ($P>0.05$), and the cytokine concentrations did not increase significantly at 12 h after LPS solution administration ($P>0.05$).

After 12 h after LPS solution addition, concentrations of IL-10, IL-12, IL17A, and INF-γ were similar to their concentrations before. Moreover, the cytokine levels did not differ in regard to glucose concentration in MC, ie, they were almost the same, regardless of the coexisting glucose concentration.

In all of the experimental groups, LPS addition led to a slight increase in TNF-α concentration but did not reach statistical significance ($P>0.05$). The level of GM-CSF rose modestly in hyperglycemia-simulating conditions, remained unchanged in hypoglycemia-simulating conditions, and fell slightly in normoglycemia-simulating conditions, but the results were not statistically significant ($P>0.05$).

The cytokine concentrations increased significantly in BC 24 h after LPS administration in BC, as presented in **Figure 6**, marked with * ($P<0.05$). Therefore, LPS must have induced an inflammatory response within the BC, despite the fact that there was no significant difference 12 h after LPS administration. The 12-h interval after LPS addition appears to be too short to observe increased concentrations of pro-inflammatory cytokines (IL-12,

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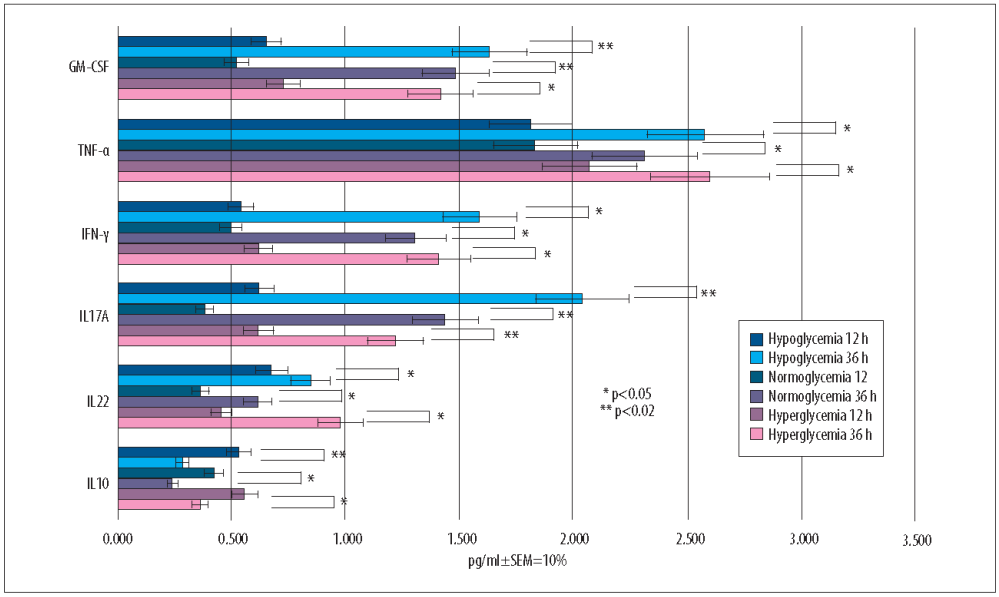

Figure 6. Mean values of cytokine concentrations (pg/ml) in brain compartment 36 h after administration of LPS compared to 12 h after LPS administration in the 3 studied groups. SEM – standard error of measurement (±10%). *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

IL17A, INF-γ, TNF-α, and GM-CSF) and decreased concentration of anti-inflammatory cytokines (IL-10).

A statistically significant cytokine level increase was found at 36 h after adding LPS to BC, marked with * (**Figure 6**). The biggest increase in cytokine concentration was for IL17A in group II (by 1.054 pg/ml; $P<0.05$), INF-γ in group III (by 0.791 pg/ml; $P<0.05$) and GM-CSF in group I and II (by 0.98 pg/ml and 0.96 pg/ml, respectively; $P<0.02$).

The statistical methods used in this part of the experiment were as described above. Statistical significance was judged at the 0.05 significance level (marked with *) or at the 0.02 significance level (marked with **) in **Figure 6**. The *P* value <0.02 indicates strong evidence against the null hypothesis, as there is less than a 2% probability the null hypothesis was correct and the results were random.

LPS Administration and Astrocyte Morphology

Astrocytes from BC after glucose and after LPS administration are presented in **Figure 7**. The 36-h culture period after setting up the model of BBB for the 3 study groups and following LPS administration did not result in changes in the morphology or viability of the astrocytes based on the day exclusion test with trypan blue. Analyzing randomly selected photos on a blind basis, experienced neuropathologists were unable to detect differences in cell morphology. Considering the above, and after additional consultation with experts in the field of histopathology, further comparative morphometric analyses were abandoned.

Anti-Inflammatory Effect of RSV in the BC

The concentrations of individual cytokines for each coexisting glucose concentration after the addition of RSV solution with a concentration of 50 μM to the MC are presented in **Figure 8**.

This part of the experiment compared the levels of 6 cytokines 24 h after applying RSV to the MC to groups without RSV and was performed separately for each glucose level.

The Mann-Whitney U test was performed and the null hypothesis was that the amount of each of the specific cytokines 24 h after applying RSV would be the same as in groups with no RSV solution. The alternative hypothesis was that the amount of each of the specific cytokines would be reduced in samples with RSV applied.

Additionally, at this stage of the study, the percentage drop of concentration levels of 6 cytokines at 24 h after RSV addition in conditions of various glucose levels was compared.

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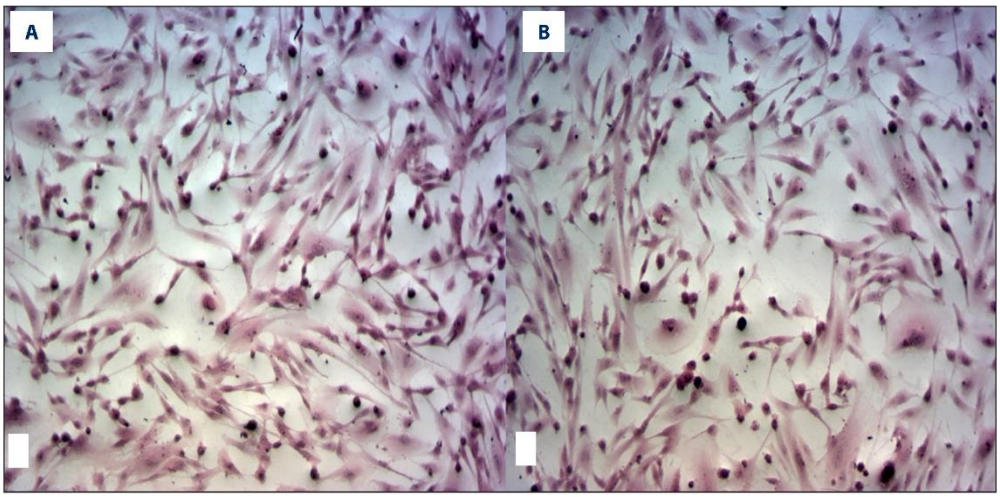

Figure 7. Astrocytes from brain compartment after glucose administration. (**A**) Staining with hematoxylin and eosin. (**B**) Astrocytes from brain compartment after glucose and LPS administration. Staining with hematoxylin and eosin. No visible changes in the morphology of the astrocytes were observed. Magnification 100×. *The picture was captured using LAS-X, the standard microscope imaging software from Leica Microsystems, Wetzlar, Germany.*

The percentage drop (effect of RSV) was based on comparison with the group without RSV solution.

The Mann-Whitney U test was performed, with the null hypothesis being that the amount of each of the specific cytokines 24 h after applying RSV would be the same regardless of glucose level. The normal glucose level is the one that was tested with the alternative hypothesis that it would generate a lower cytokine drop.

The RSV administration into MC reduced the concentration of all cytokines within the BC after 24 h regardless of glucose level, as compared to the groups without RSV, but not all the results were statistically significant (statistically significant results are marked with * in **Figure 8**).

In the normoglycemic (II) group, RSV significantly ($P<0.05$) decreased the IL-10 concentration by 0.375 pg/ml (24.2%), while in the hypoglycemic (I) group it decreased by 0.12 pg/ml (12.9%), and in hyperglycemic (III) group it fell by only 0.08 pg/ml (9%). A similar action of RSV was observed for IL-12 by decreasing its concentration by approximately 30.5% in the normoglycemic group, in comparison to the hypoglycemic and hyperglycemic groups (by 16% and 21%, respectively).

For IL-17A, the RSV level decreased most at normal glucose concentration, by 35.5%. The anti-inflammatory properties of this polyphenol were also demonstrated in groups I and III, although to a lesser extent, by 14.7% and 17.1%.

The level of IFN-γ was decreased by RSV most effectively in group II (by 20%), while in group I it fell by only 5.7% and in group III by 16%. These results were obtained within TNF-α and GM-CSF, and the greatest decline in their concentration was in group II.

The cytokines marked with * showed higher levels in the samples without RSV in comparison to those containing RSV ($P<0.05$), which suggests that RSV inhibited their production.

However, in II group the decrease of cytokine concentrations was the largest, which indicates that this polyphenol acts the most efficiently in normoglycemic conditions.

RSV Penetration Through the BBB

The concentration of RSV that reached BC after its administration to MC is presented in **Figure 9**. The results were read at 12 and 24 h after RSV addition.

In this part of the experiment, the RSV levels behind the BBB 12 h and 24 h after its application depending on the glucose level were compared. The Mann-Whitney U test for pairs was performed to check the null hypothesis that there was no difference in median of population with normoglycemia and hypoglycemia, as well as normoglycemia and hyperglycemia, with the alternative hypothesis that RSV penetration would be lower in hypoglycemia and hyperglycemia.

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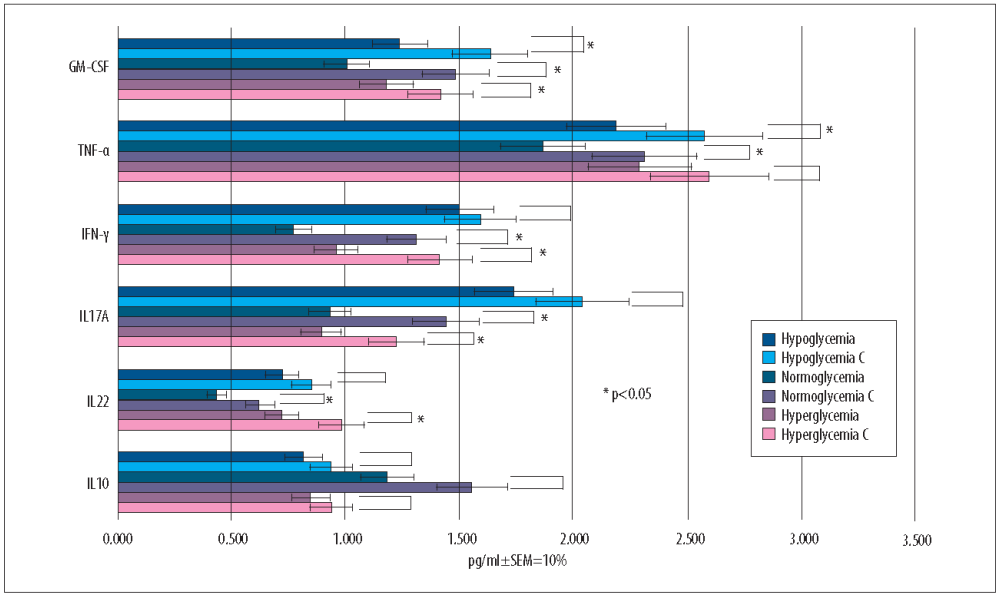

Figure 8. Mean values of cytokine concentrations in brain compartment (pg/ml) 36 h after administration of LPS and 24 h after addition of RSV to the microvascular compartment. For comparison, concentrations of the same cytokines in the samples to which LPS was added and RSV was not added (control samples are marked with the letter C). SEM – standard error of measurement (±10%). *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

The quantity of RSV administrated in MC per 200 mL of medium was 2282.5 ng. Following the instructions given with the ELISA kits and using the formula for molar concentration of a substance, the concentration of RSV which reached BC was calculated and is presented in **Figure 9**. Only 0.53% of the total amount of RSV crossed in the in vitro model of BBB in hypoglycemia-simulating conditions, 0.61% for normoglycemia, and 0.55% for hyperglycemia-simulating conditions. The findings suggest that the permeability of BBB for RSV is low, and this polyphenol can cross this semi-permeable membrane in a smaller range compared to its initial concentration.

The largest amount of RSV in BC at 12 h following its administration in MC was achieved with normal glucose concentration (69.653 ng/mL), which is 16.1% and 9.9% more than in the hypoglycemia and hyperglycemia groups, respectively ($P<0.02$).

After 24 h after RSV administration to MC, its concentration in BC in group II remained at the highest level [62.391±6.24 (ng/mL ±SEM)], significantly higher than in the other groups [group I – 42.885±4.3 (ng/mL ±SEM); group III – 48.5±4.9 (ng/mL ±SEM)], with *P* values <0.02. In a hypoglycemic environment, RSV concentration measured after 24 h decreased more rapidly than at 12 h after RSV administration, by more than 17 ng/mL. With *P* values below 0.02, the hypothesis that abnormal (both hypoglycemia and hyperglycemia) glucose levels in BC reduces the ability of RSV to penetrate the BBB was confirmed and the results were statistically significant. After 2 measurements, it was proven that abnormal glucose levels in MC (both hypoglycemic and hyperglycemia) reduces the ability of RSV to penetrate the BBB ($P<0.02$).

The main findings from the study, presenting statistically significant changes in cytokine concentrations for different glucose concentrations, are shown collectively in the **Table 3**.

Correlations Between Glucose Concentrations and Cytokine Profile

At the first stage of the study, in comparison to normoglycemia-simulating conditions, hypoglycemia-simulating conditions resulted in higher concentrations of all cytokines except TNF-α. The largest increases were for IL-12 (+89.7%), IL-17A (+56.7%), and IL-10 (+25.3%); and the increases were smaller for GM-CSF (+18.9%) and IFN-γ (+6%).

When hyperglycemia-simulating conditions were compared to normoglycemia-simulating conditions, levels of all the studied cytokines were higher. The largest increases were for IL-10 (+31.3%), GM-CSF (+28.4%), and IL-12 (+23.1%), while there

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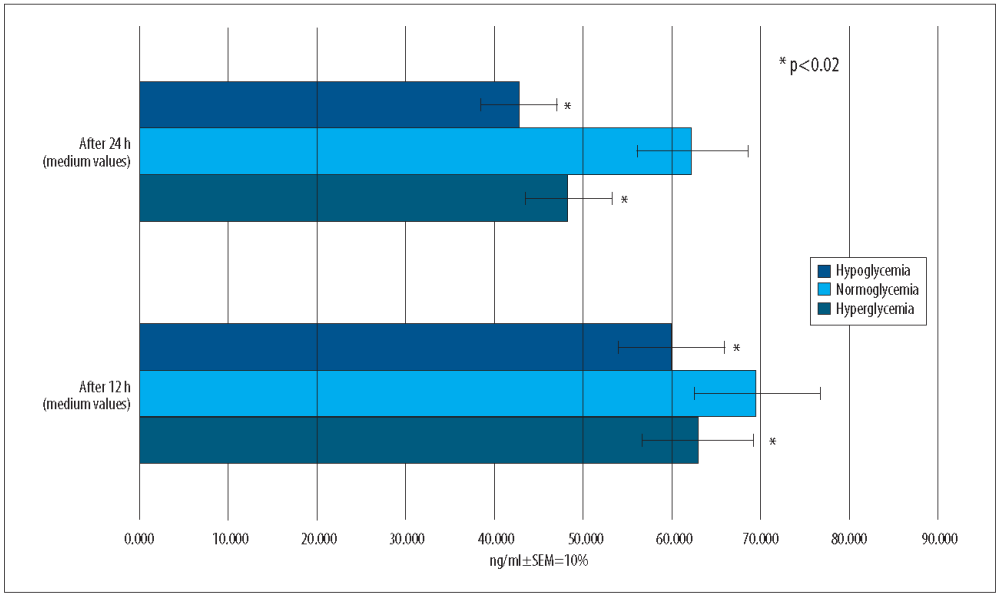

Figure 9. Mean RSV concentrations (ng/ml) in the brain compartment 12 and 24 h after RSV addition to the microvascular compartment of hypo-, normo-, and hyperglycemia-simulating samples. SEM – standard error of measurement (±10%). *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

were smaller increases for IFN-γ (+22.4%), IL-17A (+13.7%), and TNF-α (+15.7%).

The concentrations of all the studied cytokines, except TNF-α, increased both in groups with abnormally low and high glucose concentration in MC. The concentration of TNF-α was only slightly decreased in hypoglycemia-simulating conditions.

When assessing cytokine concentration at 36 h after LPS administration, IL-17A (+41.7%), IL-12 (+46.1%), IFN-γ (+21.4%), GM-CSF (+10.1%), and TNF-α (+11.5%) showed higher levels in hypoglycemia-simulating conditions than in normoglycemia-simulating conditions, while the level of IL-10 (-66.2%) changed in the opposite direction. IL-10, unlike the other studied cytokines, has anti-inflammatory properties [45,46].

When assessing cytokine concentration 36 h after LPS administration, the concentrations of IL-12 (+37.1%), IFN-γ (+7.6%), and TNF-α (+12.3%) were higher in hyperglycemia-simulating conditions than in normoglycemia-simulating conditions, while of IL-10 (-66.3%), GM-CSF (-4.5%), and IL-17A (-18.1%) changed in the opposite direction.

When assessing cytokine concentration 24 h after RSV administration, IL-10 (+31.1%) showed higher level in normoglycemia-simulating conditions than in hypoglycemia-simulating conditions, while the levels of other studied cytokines (IFN-γ [-48.7%], IL-12 [-40.2%], IL-17A [-46.6%], TNF-α [-17.5%], and GM-CSF [-20.2%]) decreased.

All studied cytokines, except IL-10 (+28.5%) and IL-17A (+2.2%), showed lower concentration in normoglycemia-simulating conditions than in hyperglycemia-simulating conditions. The largest decrease was for IL-12 (-40%), and there were smaller decreases for IFN-γ (-19.4%), TNF-α (-18.7%), and GM-CSF (-14.9%).

Discussion

In this research, a special in vitro model was prepared to imitate the topography and properties of both compartments of the BBB – the vascular side of the BBB lined with endothelial cells, and the cerebrospinal fluid side of the BBB lined with astrocytes. Commercially available in vitro models of BBB may consist of endothelial cells only, or of endothelial cells and astrocytes; some models contain not only endothelial cells and astrocytes, but also pericytes. Different categorizations of BBB models depend on the species of origin of the cells used; therefore, human, mouse, porcine, rat, and bovine models are distinguished. The overview of several BBB in vitro models developed by researchers has been presented in the most recent reviews [47-50].

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Table 3. The changes in cytokine concentrations for different glucose concentrations.

Figure number and title	Hypoglycemia	Normoglycemia	Hyperglycemia	Main findings
4. Mean values of cytokine concentrations (pg/ml) in BC, 24 h after different glucose concentrations applied in MC	Higher concentrations of GM-CSF, IL-17A and IL-12 ($p<0.05$) comparing to normoglycemia	NS	Higher concentrations of GM-CSF, TNF-α, INF-γ and IL-12 ($p<0.05$) comparing to normoglycemia	Hypoglycemia and hyperglycemia can lead to neuro-inflammation
5. Mean values of cytokine concentrations (pg/ml) in BC in 12 h after LPS has been added to BC. In comparison – concentrations of the same cytokines in LPS-free conditions	The cytokine concentrations did not increase significantly ($p>0.05$)	The cytokine concentrations did not increase significantly ($p>0.05$)	The cytokine concentrations did not increase significantly ($p>0.05$)	The inflammatory effect of LPS could not be observed as early as 12 hours after its administration
6. Mean values of cytokine concentrations (pg/ml) in BC 36 h after administration of LPS compared to 12 h after LPS administration	The concentrations of pro-inflammatory cytokines increased [IL-12, INF-γ, TNF-α ($p<0.05$)], [GM-CSF, IL-17A ($p<0.02$)] and the concentration of anti-inflammatory cytokine decreased (IL-10) ($p<0.02$)	The concentrations of pro-inflammatory cytokines increased [IL-12, INF-γ, TNF-α ($p<0.05$)], [GM-CSF, IL-17A ($p<0.02$)] and the concentration of anti-inflammatory cytokine decreased (IL-10) ($p<0.05$)	The concentrations of pro-inflammatory cytokines increased [IL-12, INF-γ, TNF-α, GM-CSF ($p<0.05$)], [IL-17A ($p<0.02$)] and the concentration of anti-inflammatory cytokine decreased (IL-10) ($p<0.05$)	The inflammatory effect of LPS was observed 36 hours after its administration
8. Mean values of cytokine concentrations in BC (pg/ml), 36 h after administration of LPS and 24 h after addition of RSV to MC	RSV reduced the level of GM-CSF and TNF-α ($p<0.05$)	RSV reduced the level of all studied cytokines ($p<0.05$), more than in other groups	RSV reduced the level of GM-CSF, INF-γ, IL-17A and IL-12 ($p<0.05$)	In the normoglycemia the anti-inflammatory effect of RSV was the greatest
9. Mean RSV concentrations (ng/ml) in the BC 12 and 24 h after RSV addition to the MC	Reduced ability of RSV to penetrate the BBB ($p<0.02$) comparing to normoglycemia	Concentration of RSV remained at the highest level, significantly higher than in the other groups ($p<0.02$)	Reduced ability of RSV to penetrate the BBB ($p<0.02$), comparing to normoglycemia	Hypoglycemia and hyperglycemia lead to decreased RSV penetration through the BBB, compared to normoglycemia

BBB – blood–brain barrier; BC – brain compartment; LPS – lipopolysaccharide; MC – microvascular compartment; NS – not significant; RSV – resveratrol.

In our study, an in vitro BBB model was created from human endothelial cells and astrocytes separated with a membrane containing 0.4-µm-wide pores, which made direct contact between different types of cells impossible. The upper limit of pore size in the BBB that enables passive flow of molecules across is <1 nm [51]. Such a diameter of pores allows diffusion of small anti-inflammatory compounds (eg, RSV) from microvascular to brain compartments [52].

In this research, the circulating blood in the microvascular system was substituted by EBM-2 medium, which did not contain cellular and non-cellular blood components (eg, leukocytes, platelets, and insulin), which is preferable to present a clear correlation between glucose concentration and cytokine production; therefore, the advantage of the in vitro model used in this study is the ability to isolate the effects of glucose concentration on cytokine production. A model consisting of 2 types of cells with no interfering factors (eg, blood and its elements or cerebrospinal fluid) allows clear results and enables investigation of specific cellular and molecular mechanisms within the BBB. By measuring the passage of glucose and cytokines across the BBB model, it is possible to gain insights into how glucose and cytokines interact with the barrier and affect its permeability. This can explain how cytokines modulate BBB responses during inflammation.

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A simplified interpretation of the results may be beneficial, showing pure relationships observed between glucose, LPS, RSV, and cytokine production by co-cultured endothelial cells in the MC and astrocytes in the BC. However, it creates an imperfect model and limits the possibility of direct translation of these results into in vivo conditions or implementation in clinical studies. The kind of model used in this study creates an inaccurate environment compared to in vivo conditions, where BBB is a highly selective and complex barrier, made not only of endothelial cells and astrocytes, but consists also of pericytes, basement membrane, and tight junctions. The McAllister study showed that glucose transport within in vivo BBB was also dependent on a serial chain of membrane-bound and intracellular transporters and enzymes, which were not present within in vitro models [53]. Although knowledge of the glucose transport and the role of glucose transporters within the BBB during systemic inflammation is still incomplete and needs further investigations, Jurcovicova's review article [54] provides a valuable description of glucose transport within the BBB.

Additionally, diabetes, as a complex metabolic abnormality, differs from hyperglycemic conditions generated experimentally. Pathologic changes in the microvascular system of diabetic patients, including abnormal angiogenesis and increased vascular density, may influence cytokine levels and RSV activity [55,56].

The results obtained in our study suggest that impaired glucose concentration induces an inflammatory response in the BC, which is reflected in the changed pro-inflammatory cytokine profiles. Among the cytokines analyzed in this study, the increase in IL-12 concentration was particularly pronounced in hypoglycemia and hyperglycemia, compared to normoglycemic environments. The increase in IL-12 concentration in hyperglycemic conditions has also been observed in a study by Huang-Pin Wu, in which the stimulated peripheral blood mononuclear cells with hyperglycemic status secreted more IL-12 than those with normoglycemic status [57]. A study by Mei-Fang Li described how high glucose increases the expression of inflammatory cytokine genes (eg, for IL-12) in macrophages through H3K9 methyltransferase mechanism [58].

Other pro-inflammatory cytokines, such as IFN-γ and GM-CSF, also reached higher concentration in the BC in conditions that simulated hyperglycemia compared to normoglycemia. Numerous studies have shown that high glucose concentrations increase the levels of those cytokines in systems other than BBB [59-61].

The existing studies indicates that both hypoglycemia and hyperglycemia can lead to modifications in pro-inflammatory cytokine profiles, which indicates inflammation [62-65]. According to Esposito et al [66], the mechanism of pro-inflammatory cytokine induction by hyperglycemia consists mainly in stimulating ROS production, because in their study, the effect was abrogated by glutathione. However, Filho et al [67] attributed glucose concentration-dependent alterations in cytokine profile observed in vivo to anti-inflammatory actions of insulin [68] and cortisol, with cortisol concentration decreasing and insulin concentration increasing during the first stage of reaction to oral glucose ingestion. Changes in the cytokine profile occur in the second stage, when insulin concentration falls, while cortisol concentration is still low, and the deficiency of anti-inflammatory actions of cortisol and insulin combined results in increased production of many pro-inflammatory cytokines. In the third stage of oral glucose tolerance testing, when cortisol concentration begins returning to normal, increased production of anti-inflammatory cytokines is observed.

In the studies mentioned above, both indirect and direct effects of changing glucose concentration towards cytokine profile have been observed. The indirect effect comprises endocrine reaction to glucose concentration, with adequately altered secretion of insulin and cortisol. The direct effect refers to activation of pro-inflammatory cytokines production by hyperglycemia, through increased glucose influx to the cells, increased rates of oxidative glycolysis and TCA, combined with a high level of intracellular ATP, which can promote ROS production through lowering the NAD+/NADH ratio in mitochondria [69].

In our study, both decreased and increased glucose concentration in MC facilitated the onset and development of an inflammatory response in BC, measured by means of increased concentrations of IL-12, IL17A, INF-γ, TNF-α, and GM-CSF.

This study was performed in vitro, so indirect, hormonal effects of altered glucose concentration towards cytokine profile can be excluded. Hyperglycemia-raised production of pro-inflammatory cytokines, if it occurs, can be attributed to pro-oxidative actions of hyperglycemia. Explaining the changes in cytokine profile in hypoglycemia-simulating conditions is more difficult; theoretically, extreme hypoglycemia can cause ATP depletion, cell necrosis, and innate immunity activation by the products of necrosis [70]. However, we did not induce cell necrosis during our study, yet we observed altered cytokine profiles in moderate hypoglycemia-simulating conditions. It can be hypothesized that either astrocytes or endothelial cells can detect changes in glucose concentration within the extracellular environment, directly or indirectly. However, it is not known whether this detection works just like in beta cells of pancreatic islets, or whether there are there any other mechanisms of signal transduction [71]. Thus, complete explanation of these mechanisms requires further research.

We decided to administer LPS in the BC because the LPS administration was supposed to develop the expected inflammatory response. Therefore, the obtained results after LPS administration can be assumed as more reliable.

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LPS as an immune system stimulator induces production of various pro-inflammatory cytokines, including TNF-α, GM-CSF, IFN-γ, IL-17A, and IL-12 [72,73].

When LPS is recognized by immune cells, such as macrophages and dendritic cells, through Toll-like receptor 4 (TLR4), it triggers a signaling cascade that leads to activation of transcription factors, such as NF-κB. This activation results in the production and release of various cytokines [74]. LPS also induces production of anti-inflammatory cytokines such as IL-10 as part of the immune response. IL-10 production is regulated by several transcription factors, including NF-κB and IRF3, which are activated by LPS stimulation [75].

The final part of the study was to assess the influence of glucose concentration on the ability of RSV to cross the BBB and on its potential anti-inflammatory effects within the CNS. In future it would be worth examining how other polyphenol substances, such as quercetin or rutin, would react to different glucose concentrations, and to evaluate their bioavailability, ability to cross the BBB, and to determine the appropriate potential dosage.

Our results show that the permeability of RSV was the greatest in the group with normal glucose concentration. In addition, the decline of RSV after 24 h was lower in this group than in the hypoglycemia and hyperglycemia groups. Hence, this flavonoid remained available for the longest time at high quantity in the normoglycemic group.

The impact of glucose concentration on RSV penetration into the brain have not been extensively studied. However, glucose concentration in blood can influence various factors that indirectly affect the transport and penetration of RSV across the BBB, and glucose concentration can affect the BBB integrity and tight junction function; thus, changes in glucose levels can impact permeability of the BBB, which in turn can influence the transport of RSV to the CNS [76,77]. Further research is necessary to understand the specific effects of glucose concentration on RSV penetration into the CNS, factors such as RSV dosage, formulation, timing of administration, and individual variations may also be significant.

Research studies indicate that despite some proven anti-inflammatory properties of RSV within CNS, its penetration through the BBB is poor [78]. RSV absorption after its oral administration can reach 75% and occurs mainly through transepithelial diffusion [79]. In addition, the metabolism of RSV in the intestine and liver results in oral bioavailability considerably less than 1% [80].

Thus, several methods are sought for to improve RSV penetration to the CSF, so that it could really exert its anti-inflammatory

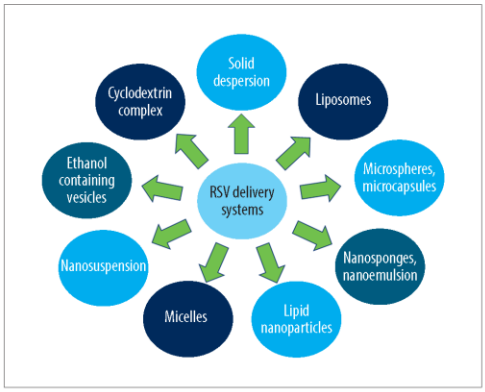

Figure 10. Resveratrol delivery systems. *Created using Microsoft® PowerPoint® 2013 (154.0.5363.1000) MSO, Microsoft Corporation.*

actions within the CNS. One of the methods is intrathecal administration of RSV. A study by Shu et al showed doubled RSV penetrability after its intrathecal administration in comparison to oral administration [81]. Another solution may be RSV molecule opsonization with some lipophilic compounds to increase its intestinal absorption, or nanoencapsulation in various structures, such as liposomes or micelles [82-84].

Yet another approach of improving RSV bioavailability for the CNS was presented by Chimento et al as modification of the RSV molecular structure, including methoxylation and hydroxylation of its aromatic rings [85]. Some novel drug delivery systems have been tested to increase the beneficial effects of RSV. Techniques used to improve RSV bioavailability are illustrated in **Figure 10**.

In the present study, RSV penetration through the BBB was taken into consideration, and further studies of cellular uptake by astrocytes could more accurately determine whether higher concentrations of RSV in the BC translate into improved use of RSV. Theoretically, based on the literature, there is no reason to doubt it, as is the case with human hepatic cells [86].

In this study, only 0.61% of the original amount of RSV was able to cross the BBB and was detected in BC in group II, while 0.53% and 0.55% crossed in the hypoglycemia and hyperglycemia groups. Despite that poor penetration, many studies suggest that even small amounts of RSV can exert beneficial effects towards the CNS due to its anti-inflammatory properties [87-89]. Furthermore, it has been shown that RSV in the CNS can slow progression of Alzheimer disease [78]. A detailed description of RSV mechanism of action in Alzheimer disease has been presented in a review [90].

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Our study has some limitations. The 6 selected cytokines were examined instead of a group of about 30 pro-inflammatory cytokines identified to date. Thus, choosing some other pro-inflammatory cytokines could elicit complementary results [91].

In addition, we found that, at least in vitro, LPS stimulates the production of different pro- inflammatory cytokines with additional latency, which should be considered when planning future in vivo studies. Naturally, endothelial cells in the co-culture also react to LPS. The assessment of the magnitude and speed of this inflammatory response may be a goal in future studies [92]. Our results suggest that further research is needed on the astrocyte response to inflammation in conditions simulating hypo-, normo-, and hyperglycemia.

Considering that different concentrations of glucose and different courses of the inflammatory reaction affect the acid–base balance, the stability and bioavailability of RSV may depend on pH. Therefore, to validating our results, the experiments could be repeated, additionally determining the pH (pH micro-measurements in culture plates) in the microvascular and brain compartments.

Neuroinflammation is a common feature of many CNS disorders, including neurodegenerative diseases (such as Parkinson and Alzheimer diseases), multiple sclerosis, and stroke, and chronic neuroinflammation can contribute to neuronal damage and disease progression. Thus, it is crucial to reduce the harmful effects of chronic neuroinflammation by finding effective anti-inflammatory strategies. RSV, with its proven properties, can modulate various signaling pathways involved in inflammation. It can suppress the production of inflammatory cytokines (eg, TNF-α, IL12, and IL-17) [93,94], inhibit the activation of pro-inflammatory transcription factors (eg, NF-κB) [95], and as an anti-oxidative agent RSV can help reduce oxidative damage and protect neurons from free radicals and ROS [96,97], which are often present in neuroinflammation.

By conducting in vitro experiments, as in our study, it is possible to gain insights into the impact of glucose concentration on RSV's ability to penetrate and cross the BBB. The mechanisms of RSV uptake by astrocytes are still being studied and there may be many pathways involved. The primary mode of RSV uptake is passive diffusion; due to RSV's lipophilic nature, it can passively diffuse across cell membranes, as in astrocytes, and studies have suggested the involvement of transporters in its cellular uptake by astrocytes. Organic anion-transporting polypeptides (OATPs) and ATP-binding cassette (ABC) transporters have been implicated in the uptake of RSV in various cell types, including astrocytes [98-101]. These transporters may facilitate the entry of RSV into astrocytes, potentially increasing its uptake efficiency. When RSV is taken up by astrocytes, it can be distributed within different cellular compartments. It has been found in the nucleus, cytoplasm, and mitochondria of astrocytes, which suggest its potential interactions with various cellular components and signaling pathways [102,103]. In addition, RSV has been reported to enhance synaptic plasticity, which plays a role in learning and memory processes, and it can expedite the formation of synaptic connections by modulation of neurotransmitter systems and neurotrophic factors [104,105]. Some studies suggest that RSV can also influence BBB integrity and function, which regulates the entry of substances into the brain. Thus, RSV may influence drug delivery to the CNS and thus modulate some CNS disorders [106,107].

It is essential to further explore RSV's properties by conducting preclinical studies to clarify the mechanism of action, optimizing dosage regimens, and evaluating its efficacy and safety in relevant animal models. Then, clinical trials are needed to assess this potential in patients with CNS diseases.

Conclusions

In our experiment, RSV decreased the concentrations of pro- and anti-inflammatory cytokines in all 3 study groups, regardless of the glucose concentrations, but exerted the strongest anti-inflammatory properties in a normoglycemic environment.

The strength of those properties depended not only on the glucose concentration in the MC but also on the cytokine studied. The strong effects of RSV were obtained for IFN-γ and TNF-α, which is compatible with the findings of Torregrosa-Muñumer from an in vivo study performed on old male rats, where the expression of 2 major inflammatory markers, INF-γ and TNF-α, increased with aging, but were subsequently reduced to the levels observed in hearts of young animals after RSV supplementation [108].

Our results show it is essential to maintain normoglycemia, since the anti-inflammatory effects of substances such as RSV can be most effectively exerted under normoglycemic conditions. Furthermore, prolonged hyperglycemia induces ROS production in astrocytes and pericytes, which additionally increases CNS inflammation [109].

Accordingly, supplementation of this flavonoid in diabetic patients is worth considering. *Further in vivo* studies are required to assess its beneficial anti-inflammatory effects, especially in patients with abnormal glucose concentrations. RSV shows beneficial effects that are worth further studying and taking advantage of.

Declaration of Figures' Authenticity

All Figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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6.3.

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Article

Anti-Inflammatory Action of Resveratrol in the Central Nervous System in Relation to Glucose Concentration—An In Vitro Study on a Blood–Brain Barrier Model

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Abstract: Unbalanced blood glucose levels may cause inflammation within the central nervous system (CNS). This effect can be reversed by the action of a natural neuroprotective compound, resveratrol (RSV). The study aimed to investigate the anti-inflammatory effect of RSV on astrocyte cytokine profiles within an in vitro model of the blood–brain barrier (BBB) under varying glucose concentrations (2.2, 5.0, and 25.0 mmol/L), corresponding to hypo-, normo-, and hyperglycemia. The model included co-cultures of astrocytes (brain compartment, BC) and endothelial cells (microvascular compartment, MC), separated by 0.4 µm wide pores. Subsequent exposure to 0.2 µM LPS in the brain compartment (BC) and 50 µM RSV in the microvascular compartment (MC) of each well was carried out. Cytokine levels (IL-1 α, IL-1 β, IL-2, IL-4, IL-6, IL-8) in the BC were assessed using a Multi-Analyte ELISArray Kit before and after the addition of LPS and RSV. Statistical analysis was performed to determine significance levels. The results demonstrated that RSV reduced the concentration of all studied cytokines in the BC, regardless of glucose levels, with the most substantial decrease observed under normoglycemic conditions. Additionally, the concentration of RSV in the BC was highest under normoglycemic conditions compared to hypo- and hyperglycemia. These findings confirm that administration of RSV in the MC exerts anti-inflammatory effects within the BC, particularly under normoglycemia-simulating conditions. Further in vivo studies, including animal and human research, are warranted to elucidate the bioavailability of RSV within the central nervous system (CNS).

Keywords: blood–brain barrier; astrocytes; glucose; resveratrol; inflammatory response modulation; anti-inflammatory action

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1. Introduction

Impaired glucose tolerance (IGT) and diabetes occur worldwide on a pandemic scale, which is promoted by increased metabolic disorders in addition to a rise in obesity levels. Because nervous cells preferentially use glucose to obtain energy, and the influx of glucose to neurons is not insulin dependent [1], oscillations of glucose concentration can affect metabolic processes within the CNS. Both diabetes-related hyperglycemia and iatrogenic hypoglycemia (induced by anti-diabetic insulin treatment) may affect the functioning of the CNS [2,3]. Inflammation in the CNS can damage the neurons, as was documented with Alzheimer's disease, Parkinson's disease, encephalitis, meningitis, neurosarcoidosis, and hyperglycemia [3–9]. Neuroinflammatory processes can be particularly difficult to predict in diabetes because the cytokine profile in the CNS depends on serum glucose concentration [10]. High prevalence of neuroinflammation-associated diseases impairs the

functioning of millions of people all over the world. Therefore, many research studies aim at finding a remedy for neuroinflammation, including diabetes-associated neuroinflammation.

Hyperglycemia can result in an increased glucose influx to BBB-forming cells (both pericytes and astrocytes) [3,11]. Temporary, incidental high blood sugar levels are not harmful to neurons, because the passage of glucose into the cerebrospinal fluid is tightly regulated by a healthy BBB, but prolonged periods of hyperglycemia in diabetic patients can lead to the production of reactive oxygen species (ROS) in astrocytes and pericytes, primarily due to an accelerated process of aerobic oxidative glycolysis, followed by subsequent reactions in the tricarboxylic acid cycle that convert oxidized nicotinamide-adenine dinucleotide (NAD+) into its reduced form, NADH [12–14]. It should be noted that neither NAD+ nor NADH can cross the mitochondrial membrane, so mitochondrial NAD+ depletion cannot be neutralized by increased influx of NAD+ from other cell compartments and excess NADH cannot be eliminated from the mitochondrion through simple diffusion [15,16]. Electrons carried by NADH are transferred to the electron transport chain (ETC) to produce ATP, but this process can be ineffective when the ATP level in the cell is already high. In such circumstances, NADH can transfer electrons non-enzymatically to molecular oxygen, which results in ROS production [17]. Elevated levels of ROS in astrocytes and pericytes may trigger the activation of nuclear factor-κappa B (NF-κB), leading to inflammation and an increase in vascular permeability [18–20]. At this stage, glucose influx into the CNS ceases to be strictly controlled, which results in increased glucose influx into neurons [20,21], and induces an inflammatory response in neurons through activation of the NF-κB pathway described above. In addition, mtDNA damage by increased amounts of ROS can induce production of chaperon proteins, such as HSP-60 [22]. Some HSP-60 molecules can be transferred to other cell compartments, such as cell membranes, where they can activate pattern recognizing receptors (PRRs), which can, in turn, activate innate immunity-related inflammation [23]. Additionally, innate immunity can be also activated by advanced glycation products (AGEs) [24].

Since the brain does not produce glucose or store substantial glycogen reserves within astrocytes, it depends on a continuous influx of glucose from the bloodstream [25]. Consequently, hypoglycemia instigates the depletion of cerebral energy reservoirs, leading to cognitive malfunction of the brain, culminating in coma or death [26]. There is a plausible link between recurring instances of profound hypoglycemia and enduring cognitive impairment [27]. While the precise mechanism remains elusive, it has been documented that hypoglycemia leads to elevated levels of inflammatory cytokines and an increase in white blood cell count, indicating a potential association between low blood sugar and inflammation [28,29]. In the recently published study on induction of hypoglycemia in type 2 diabetes (T2D), the levels of both inflammation and oxidative stress markers were significantly increased in urine and blood samples at 24 h following the hypoglycemic episode in humans. This effect was not observed in T2D-free controls [30]. The above-described proinflammatory effects of metabolic disturbances in the CNS accompanying hyperglycemia and hypoglycemia are presented in Figure 1.

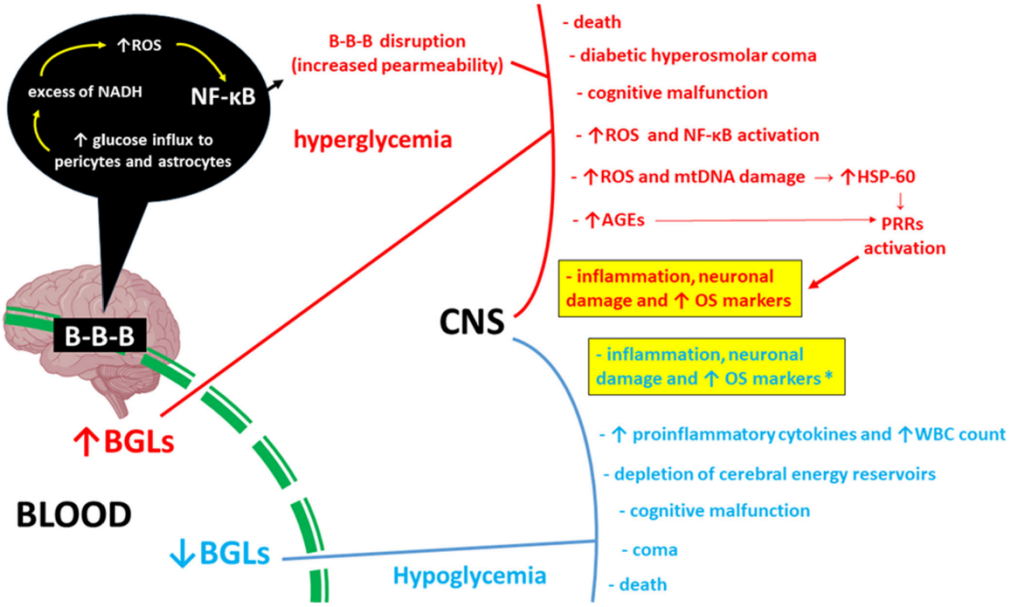

Figure 1. Pro-inflammatory consequences of hyper- and hypoglycemia (marked in red and blue, respectively) in the central nervous system (CNS). Increased glucose inflow into pericytes and astrocytes in hyperglycemia leads to excess reduced nicotinamide adenine dinucleotide (NADH), which results in excessive reactive oxygen species (ROS) production and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [3,11,17]. This damages the integrity of the blood–brain barrier (B-B-B) and increases its permeability [18–20]. Disturbance of glucose homeostasis in the cerebral compartment leads to the induction of inflammation in a similar mechanism. Additionally, mitochondrial DNA (mtDNA) damage is accompanied by an increase in the production of the chaperone protein HSP-60, which can activate pattern recognition receptors (PRRs) and subsequently induce innate immunity-related inflammation [23]. Advanced glycation end products (AGEs) also activate PPRs [31]. CNS inflammation in iatrogenic hypoglycemic states in type 2 diabetes (T2D) is accompanied by increased concentrations of proinflammatory cytokines and elevated white blood cell (WBC) count. Both hyper- and hypoglycemia, inflammation, and increased concentrations of oxidative stress (OS) markers are accompanied by clinical symptoms of varying severity [32]. ** in relation to iatrogenic hypoglycemia in T2D; ↑ BGLs—increased blood glucose levels; ↓ BGLs—decreased blood glucose levels.*

Resveratrol, a polyphenol naturally found in the skin of red grapes and berries, is known for its role in mitigating chronic inflammatory conditions [33]. Beneficial effects of RSV have been recently documented, e.g., in a diabetic elderly female rat model, in which RSV treatment resulted in cardioprotective activity [34,35]. The anti-inflammatory and antioxidant characteristics of RSV are associated with its ability to activate sirtuins (SIRTs) either directly or indirectly [36]. SIRTs activation occurs through allosteric modification of their molecules or by increase in the expression and activity of nicotinamide phosphoribosyl transferase (NAmPRTase or NAMPT) and AMPK [37,38]. SIRTs represent a family of enzymes, primarily functioning as NAD^+-dependent deacetylases, consisting of seven members in humans and other mammals [39,40]. Activation of SIRTs may be responsible for neuroprotective effects of RSV that correspond to epigenetic inhibition of the p53 protein and epigenetic increase in the expression of peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) [38,41,42]. Overall, the bioactivity of RSV as the

neuroprotective agent includes inhibition of the NF-κB signaling pathway, activation of autophagy instead of apoptosis, and modulation of the key activity of mitogen-activated protein kinases (P38-MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphoinositide 3-kinase (PI3K)/Akt. [38,43]. Regardless of the not yet fully understood mechanisms of action, it has been confirmed in many research studies, performed mainly on in vivo models, that RSV shows beneficial effects on cognitive processes and memory, as well as on hippocampus connectivity and microstructure in older adults [44–47].

The BBB comprises a unique composition of circulatory system and CNS. It protects neurons from potentially harmful substances through passive and selective transport of ions, molecules, and cells from the blood to the cerebrospinal fluid (CSF) [48]. Endothelial cells within the BBB are strictly connected with occlusion zones, without fenestrations [49]. The maximum pore size within the BBB that allows for the passive flow of molecules is typically below 1 nm in diameter [50]. Therefore, the larger substance transport from the plasma occurs transcellularly through facilitated diffusion or the protein transport system (PTS) [51].

Thus, not all substances can cross the BBB and enter the CNS. Only molecules of molecular weight below 500 Da can effectively penetrate the BBB [52–54]. Numerous anti-inflammatory compounds are under investigation for their ability to cross the BBB and to reach the CNS. RSV, due to its low molecular weight of 228 Da and its lipid-soluble properties, can effectively cross the BBB [55].

Before the substances become available to neurons, they are transported into astrocytes [56]. In the human brain, astrocytes are much more numerous than neurons, and they have essential functions within the CNS, including regulation of the level of substances like glutamate, ions (such as calcium and potassium), and water [57]. Astrocytes also protected against damage from oxidative and nitrosative stress, store energy, assist in generating mitochondria, contribute to scar formation, aid in tissue repair through processes like angiogenesis and neurogenesis, and modulate synapses [58]. Additionally, as part of the BBB, astrocytes are strategically positioned between the blood vessels in the brain and the connections between neurons, allowing them to facilitate the uptake of nutrients from the bloodstream [59].

In the cytoplasm of the endothelial cells and astrocytes, substances are enzymatically processed to constitute another biochemical component of the BBB [60]. Interestingly, direct cell-to-cell contact between astroglial cells and brain capillary endothelial cells is the necessary precondition for normal enzymatic activities of gamma-glutamyltranspeptidase (γ-GTP) and alkaline phosphatase (ALP), the enzymes commonly used as markers to evaluate the BBB's characteristics [61].

Due to the dependence of neuroinflammation on the body's metabolic state, it is important to investigate whether the metabolic status, particularly in terms of plasma glucose levels, can alter the anti-neuroinflammatory effects of RSV. This study is a continuation of the analysis of changes in the cytokine profile under the influence of RSV at different glucose concentrations, based on an in vitro BBB model, the results of which have already been published in relation to interleukins (IL-10, IL-12, IL-17A), tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [62]. The current analysis considers other cytokines (interleukins) with model neuroinflammatory effects (IL-1α, IL-8) [63,64], pro-inflammatory and neurodifferentiating potential (IL-1β) [65,66], neuro-immune-modulatory properties (IL-2) [67,68], and anti-inflammatory or neurotrophin-like activities within the CNS (IL-4, IL-6) [69–71].

2. Results

2.1. Co-Cultured Cells Viability and Morphology

Throughout the entire 36 h incubation period following the creation of the BBB model for the three study groups under hypo-, normo-, and hyperglycemic conditions, the consecutive intervals of 36 h after administering LPS had no significant impact on the vitality of co-cultured cells. This assessment was based on the results of the exclusion test using

trypan blue and the observation of co-cultured cell morphology. Astrocytes after glucose and LPS administration are presented in Figure 2.

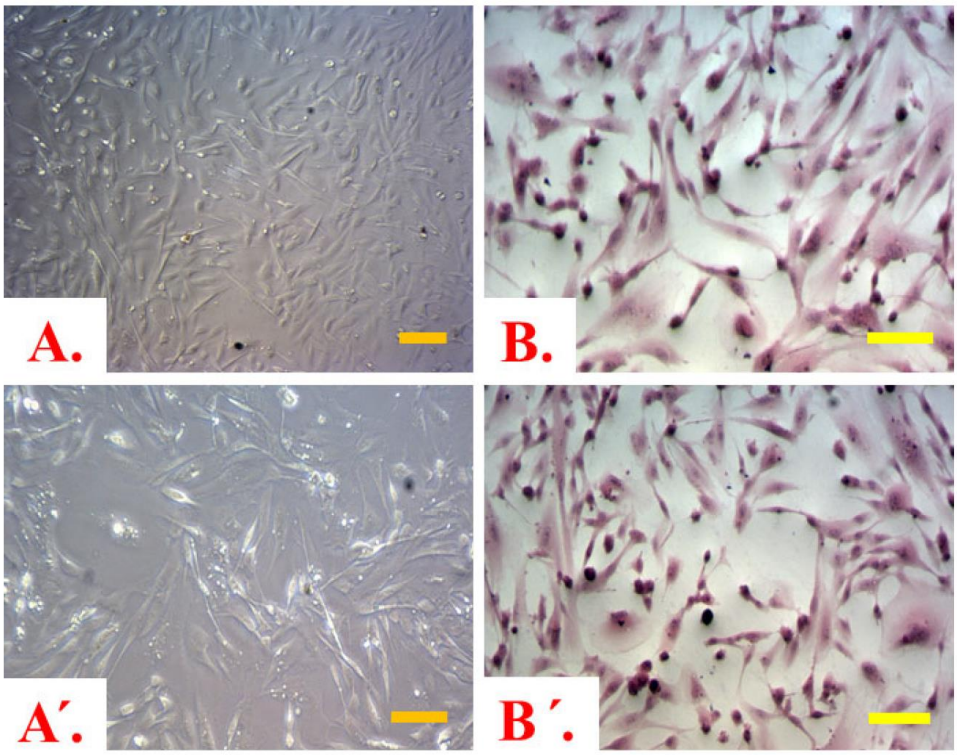

Figure 2. Astrocytes from the brain compartment (BC): (**A**,**A′**)—after glucose serum added and after 0.2 µM LPS solution added, respectively—no staining; (**B**,**B′**)—astrocytes after glucose serum added and after 0.2 µM LPS solution added, respectively—staining with hematoxylin and eosin (H&E). Despite different glucose concentrations used, no significant changes in astrocyte's morphology have been observed (based on analysis of slides stained with H&E). Scale bars = 50 µm.

2.2. *Effect of Glucose Concentration in MC on Inflammatory Response within BC*

The mean concentration of measured cytokines in the samples obtained within the three studied groups (hypo-, normo-, and hyperglycemic) from the BC after 24 h following glucose addition, which may correspond with CSF in the BBB model used, are presented in Figure 3.

The concentration of IL-1α in the samples from a high-glucose environment (group III) was the highest, measuring 0.915 ± 0.09 (pg/mL ± SD), while slightly lower values (0.89 ± 0.09) were observed in samples collected from a low-glucose environment (group I). Notably, the normoglycemic group (group II) exhibited the lowest mean IL-1α level, with a value of 0.58 ± 0.06, which was significantly different ($p < 0.05$) from the mean values in both group I and group III.

The levels of IL-1β and IL-2 were not affected by different glucose concentrations, and the differences for these cytokines in hypo-, normo-, and hyperglycemia were not statistically significant.

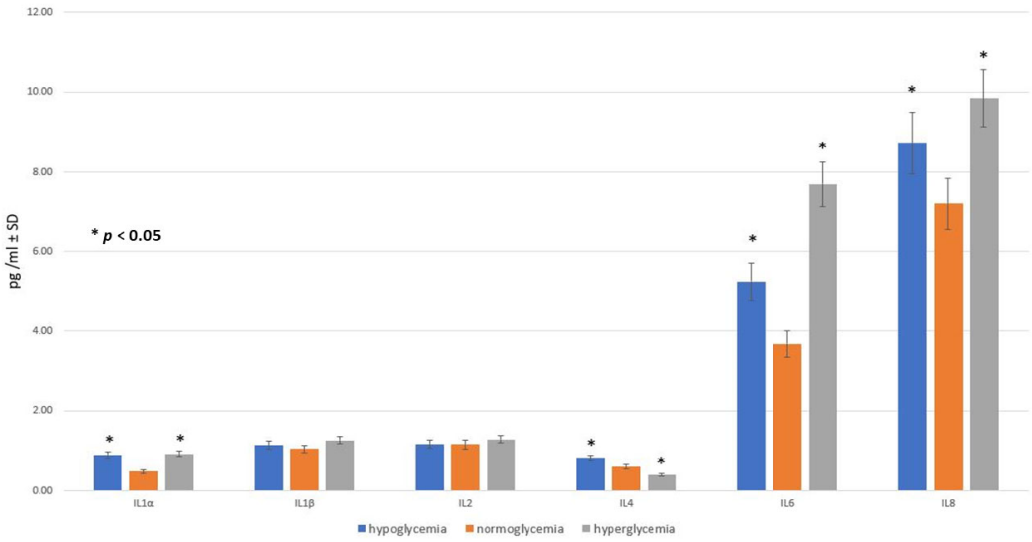

Figure 3. The cytokine concentrations in the brain compartment after 24 h following glucose addition to obtain hypo-, normo-, and hyperglycemic environments: mean values ± standard deviation (pg/mL ± SD). Mann–Whitney U test (n = 6 records for each cytokine within each group).

Interestingly, and unlike for the other studied cytokines, in the cytokine profile of IL-4, the highest level of IL-4 was observed in conditions imitating hypoglycemia, whereas the lowest IL-4 concentration was measured in the samples obtained from the hyperglycemic environment. These values significantly differed ($p < 0.05$) from the IL-4 level obtained for normoglycemia (see Figure 3).

Leaving the cytokine concentrations, the profiles for IL-6 and IL-8 changed similarly, with statistically significant ($p < 0.05$) differences between the groups. Here, the lowest mean values were observed in the normoglycemic environment, intermediate levels in hypoglycemia, and the highest levels in hyperglycemia. The biggest difference measured in samples containing different glucose concentrations was shown within the IL-6 values, which amounted to 3.67 ± 0.37 (pg/mL ± SD) for normoglycemia and were increased to 7.68 ± 0.77 in the hyperglycemia-mimicking environment.

There was a big difference measured in the samples containing different glucose concentrations for the IL-8 values, which equaled about 7.2 ± 0.72 (pg/mL ± SD) when the glucose concentration was normal, while they rose to 9.84 ± 0.99 when the glucose concentration was high. This is in accordance with the existing results of other studies showing that hyperglycemic conditions increase the concentration of IL-8 in systems other than the BBB, such as in human gingival epithelial cells [72] or in keratinocytes [73], among others. It is worth noting that the results in the control group without glucose were undetectable.

2.3. Effect of LPS Concentration on Inflammatory Response within BC

Administration of 0.2 µM lipopolysaccharide (LPS) solution into the BC caused a wide variety of changes in the cytokine profiles within 12 h compared to the controls (Figure 4).

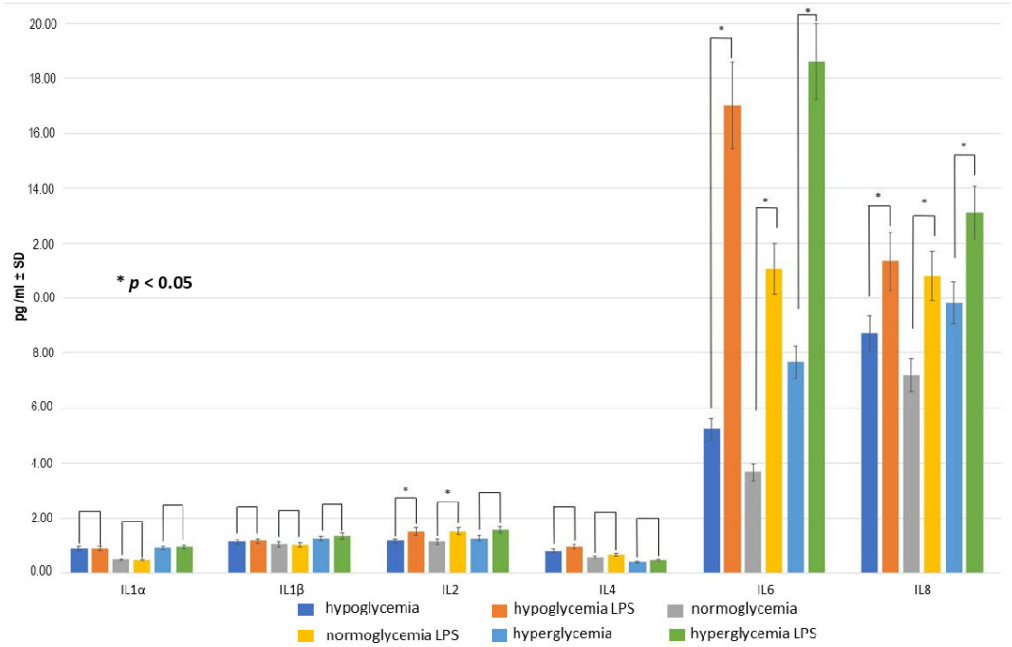

Figure 4. The cytokine concentrations in the brain compartment (BC) after 12 h following 0.2 μM LPS solution administration in BC in hypo-, normo-, and hyperglycemic environments (with 24 h duration of exposure to different glucose concentrations before LPS administration) compared to the respective LPS-free environments at the beginning of experiment: mean values ± standard deviation (pg/mL ± SD). Mann–Whitney U test (n = 6 records for each cytokine within each group).

The levels of IL-1α, IL-1β, and IL-4 in groups I–III were similar to those in the respective control samples, indicating a lack of LPS-induced inflammatory response, whereas this response was observed for IL-6 and IL-8 with significant ($p < 0.05$) increases in these cytokine levels in all three examined groups. Moreover, for IL-6, the differences between both hypoglycemic + LPS and hyperglycemic + LPS subgroups versus normoglycemic + LPS subgroup were also significant.

In the case of IL-2, the levels of the cytokine were indeed significantly increased after 12 h following LPS administration in group I and II (hypoglycemic and normoglycemic environment, respectively), but in hyperglycemic conditions (group III), the differences did not reach statistical significance.

2.4. Effect of RSV Administration to MC on Inflammatory Response within BC

After 36 h following LPS administration, the concentrations of the studied pro-inflammatory cytokines increased significantly ($p < 0.05$) in the majority of cases compared to the respective concentrations after 12 h (Figure 5). This was true for hypo-, normo-, and hyperglycemia, with a few exceptions: there was a lack of statistical significance between observed differences for IL-1α, IL-2 and IL-4 in the hypoglycemic environment and non-significant differences for IL-2 in hyperglycemia.

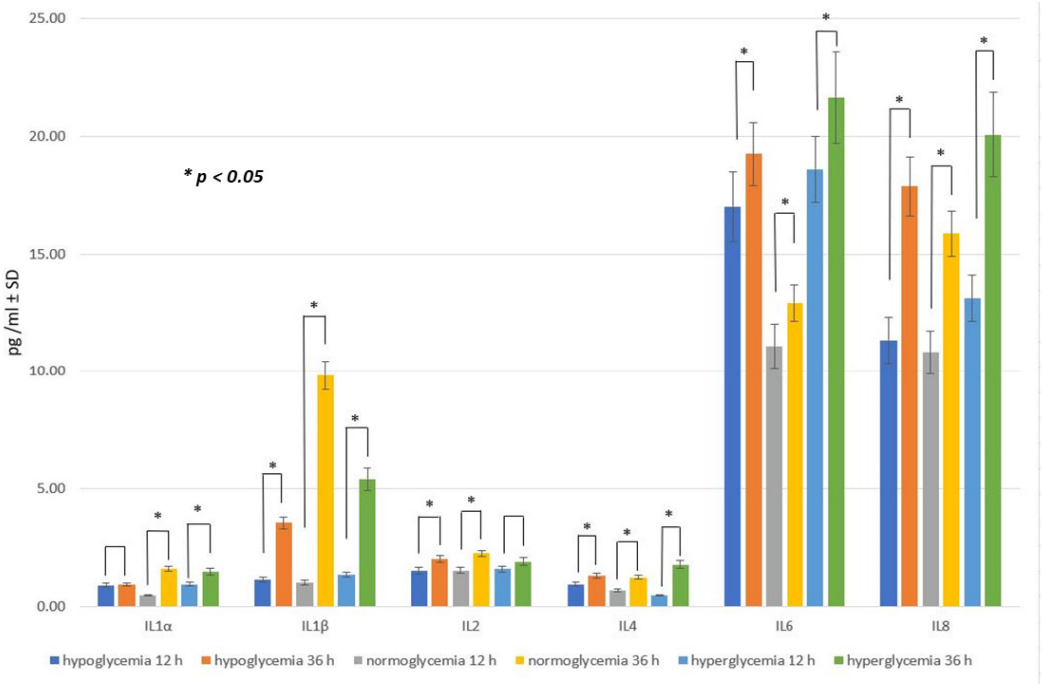

Figure 5. The cytokine concentrations in the brain compartment after 36 h following 0.2 μM LPS solution administration in BC in hypo-, normo- and hyperglycemic environments (with 24 h duration of exposure to different glucose concentrations before LPS administration) compared to the respective values obtained 12 h after 0.2 μM LPS administration: mean values ± standard deviation (pg/mL ± SD). Mann–Whitney U test ($n = 6$ records for each cytokine within each group).

The effects of RSV administration on the cytokine profiles at different glucose concentrations are presented in Figure 6.

In this stage of the experiment, separate tests for each glucose environment to compare the levels of six different cytokines 24 h after applying RSV to the cells in two groups: one with RSV and one without, were conducted. The Mann–Whitney U test for this comparison was used.

The null hypothesis for this test stated that the concentration of each specific cytokine would be identical 24 h after applying RSV in both the RSV group and the group without RSV, while the alternative hypothesis suggested that the presence of RSV would lead to a decrease in the concentration of these cytokines.

Furthermore, the percentage reduction in cytokine concentration levels at 24 h after administering RSV in three different study groups was assessed. The percentage drop (representing the effect of RSV) was determined by comparing each group to the one without the RSV solution.

The Mann–Whitney U test was used for this comparison as well. The null hypothesis for this test posited that the concentration of each specific cytokine 24 h after applying RSV would be the same regardless of the glucose level, with the alternative hypothesis suggesting that the normal glucose level would result in a greater reduction in cytokine levels.

The introduction of RSV into the MC led to a decrease in the levels of all cytokines in the BC after 24 h, irrespective of glucose levels, when compared to the groups that

did not receive RSV. However, it is worth noting that not all of these changes reached statistical significance.

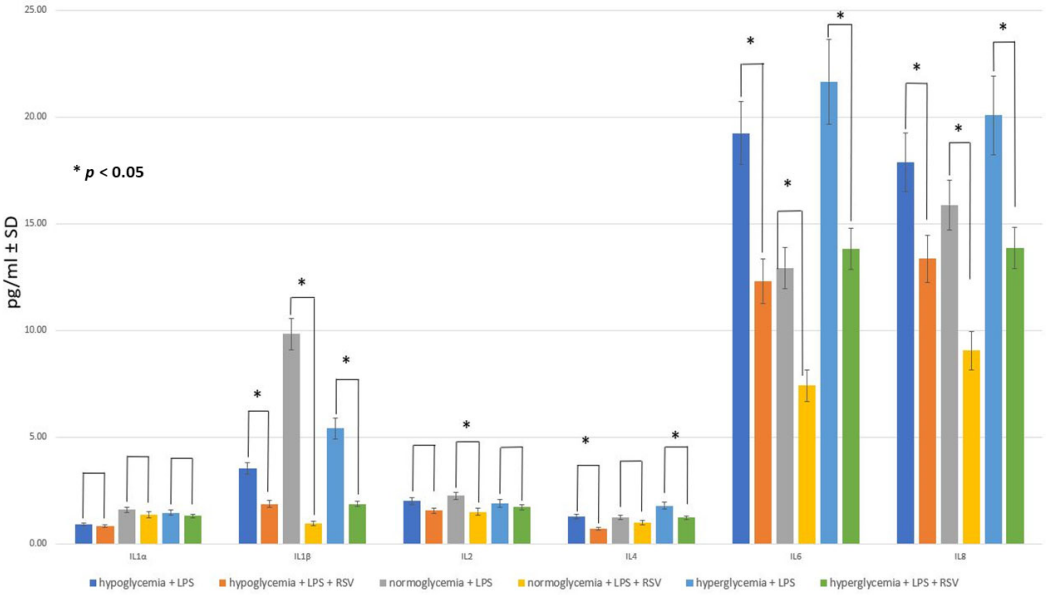

Figure 6. The cytokine concentrations in the brain compartment after 24 h following 50 μM resveratrol (RSV) addition (and after 36 h following 0.2 μM LPS solution adding) into hypo-, normo-, and hyperglycemic environments compared to the respective values obtained after 36 h following 0.2 μM LPS solution addition without subsequent administration of RSV: mean medium values ± standard deviation (pg/mL ± SD). Mann–Whitney U test (n = 6 records for each cytokine within each group).

RSV decreased the concentration of IL-1α by approximately 10%, regardless of glucose concentration, but the results were not statistically significant ($p > 0.05$). For IL-1β, this action of RSV was statistically significant; however, it was much stronger at normal glucose concentrations, where a major reduction in this cytokine was observed (the decrease in the IL-1β level was estimated to be 10 times), compared to hypoglycemia- or hyperglycemia-mimicking conditions, where the IL-1β concentration was 2 to 3 times lower after RSV administration.

RSV significantly decreased IL-2 concentration only in normoglycemia-simulating conditions, by 29%. The results for IL-4 suggest that the optimal action of RSV towards the decreasing IL-4 level took place in hypoglycemic- and hyperglycemic conditions but not in normoglycemia.

The levels of IL-6 and IL-8 decreased significantly 24 h after RSV administration into the MC. For IL-6, RSV reduced its concentration in hypo-, normo-, and hyperglycemic conditions by 42%, 53%, and 49%, respectively, and for IL-8 by 25%, 43%, and 31%. These results are in agreement with other studies that documented the ability of RSV to decrease both IL-6 and IL-8 levels [74–76].

Thus, the preliminary assumption that RSV added to MC reduces the concentration of cytokines in the BC after 24 h of application in the experimental environment regardless of glucose level was confirmed with statistical significance for measurements marked with an asterisk (*) in Figure 6. The largest decrease for IL-1β, IL-6, and IL-8 was observed in normoglycemia-simulating conditions compared to both hypo- and hyperglycemia ($p < 0.05$).

Referring to the numerical data in Figure 6, RSV administration in hypoglycemia-simulating conditions resulted in higher concentrations of IL-1β (+48.3%), IL-2 (+3.6%), IL-6 (+40%), and IL-8 (+32.2%), compared to the normoglycemic conditions, whereas the levels of other cytokines studied, i.e., IL-1α and IL-4, were lower in hypoglycemia- than in normoglycemia-mimicking conditions (−38.5% and −27%, respectively).

RSV administration in hyperglycemia-simulating conditions resulted in higher concentrations of IL-1β (+48.5%), IL-2 (+12.3%), IL-4 (+19.4%), IL-6 (+46.3%), and IL-8 (34.7%), compared to the normoglycemic conditions, while the level of IL-1α was lower in the hyperglycemic than in normoglycemic conditions (−4.7%).

2.5. RSV Penetration through the BBB Model Applied

An examination of RSV transfer between the two compartments through the semi-permeable membrane of the BBB model reveals that RSV exhibits limited bioavailability. Specifically, when 2282.5 ng of RSV was administered to the MC in a 200 µL (0.2 mL) volume, the resulting concentration of RSV in the MC was 11,412.5 ng/mL. Employing a well-established formula for calculating molar concentration and adhering to the instructions provided with the ELISA kits, the concentration of RSV in the BC was determined. The findings from these ELISA measurements and subsequent computations are presented in Figure 7. RSV concentrations in the BC are significantly lower than the initial amount administered in the MC for each glycemic condition.

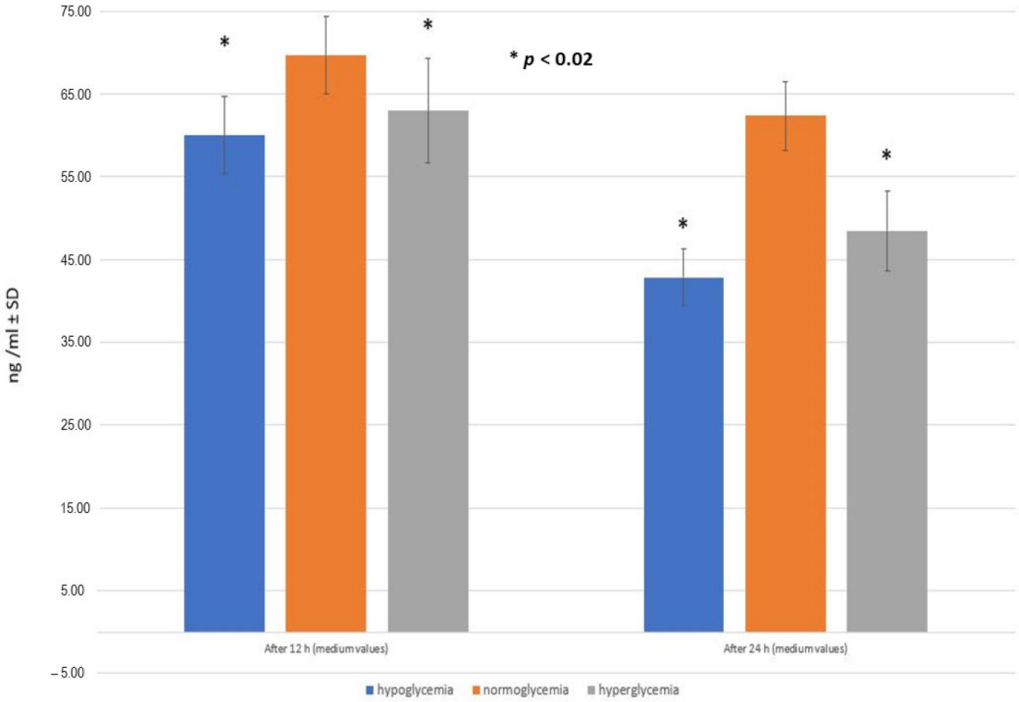

Figure 7. The mean concentration of resveratrol (RSV) in the brain compartment after 12 h and after 24 h following RSV adding into hypo-, normo-, and hyperglycemic environments of the microvascular compartment (ng/mL ± SD). Mann–Whitney U test (n = 6 records for each timepoint within the groups).

In this section of the experiment, the Mann–Whitney U test for paired samples was employed to assess the null hypothesis, which posited that there were no distinctions in the mean values of RSV concentration between populations exhibiting normoglycemia and hypoglycemia, as well as normoglycemia and hyperglycemia. Conversely, the alternative hypothesis posited that RSV would exhibit different levels in the BC in hypoglycemic and hyperglycemic conditions, in regard to normoglycemia. Each determination was conducted six times, and the outcomes are reported as mean values accompanied by their corresponding standard deviations.

After conducting six measurements, it was observed that the concentration of RSV in the BC was higher in normoglycemia- (0.61% of the initial concentration) compared to hypoglycemia- and hyperglycemia-mimicking conditions (0.53% and 0.55% of the initial concentration, respectively).

In the normoglycemic condition, even after 24 h of administering RSV to the MC, its concentration in the BC remained consistently high, significantly higher than in other groups (p-value < 0.02). In the hypoglycemic and hyperglycemic environment, the concentration of RSV measured 24 h after administration was significantly lower compared to the measurement taken after 12 h, dropping by more than 17 ng/mL and by 15 ng/mL, respectively.

These results confirm the initial hypothesis that abnormal blood glucose levels can significantly impair RSV availability in the CNS.

3. Discussion

Numerous variants of the BBB models have been created by different teams of researchers, with some being commercially available [77]. Diverse BBB models encompass various cell combinations, such as co-cultured pericytes, astrocytes, and endothelial cells, astrocytes with endothelial cells, or solely endothelial cells. Furthermore, in the realm of predicting BBB permeability, models can be categorized into monoculture, non-contact co-culture, and contact co-culture systems. Another classifying factor for BBB models is the species of origin of the cells applied, which includes animal models (e.g., mouse, rat, bovine, or porcine) and human models [78,79].

In our study, the BBB model was set up from human astrocyte and endothelial cell lines, to imitate conditions occurring on both sides of the barrier—namely, BC and MC. The advantage of such a model is the selectivity regarding the permeation test that is not influenced by cellular and non-cellular blood components or CSF [80]. On the other hand, such an approach and the use of cell lines has certain limitations, especially with regard to in vivo conditions.

The obtained results indicate that the abnormal level of glucose in the MC are already a trigger of an inflammatory reaction in the BC, reflected by the increased level of pro-inflammatory cytokines. It is important to note that both low and high blood sugar environments can cause inflammation, as has been observed in previous research studies [81,82].

Among the cytokines analyzed in this study, the levels of IL-8 and IL-6 were significantly increased in the BC in hyperglycemia, compared to both normoglycemic and hypoglycemic environments (groups I and II, respectively). IL-8, also known as neutrophil chemotactic factor or chemokine CXCL8, is a key mediator associated with the immune reaction in the innate immune system response [83]. In relation to IL-6, a cytokine reported to exhibit both inflammatory and anti-inflammatory characteristics, astrocytes are identified as a significant source of this interleukin [84]. While IL-6 can be beneficial in the CNS due to its ability to promote nervous cell growth, an excessive production of IL-6 can be detrimental, contributing to the pathophysiology of CNS disorders [85]. It has been observed that high glucose induces the expression of IL-6 in cultured astrocytes [86].

Additionally, pro-inflammatory cytokines like IL-1α, IL-1β, and IL-2 attained their peak levels in the BC under conditions that mimicked high blood sugar, in contrast to normal blood sugar levels. Previous research, conducted in various systems beyond the

BBB, has consistently demonstrated that elevated glucose concentrations lead to an increase in these cytokines [87–90]. IL-1β is a key immunoregulatory and pro-inflammatory cytokine and its increase is acknowledged as a vital element in the brain's structured reaction to neuroinflammation and in the recruitment of white blood cells to the CNS [91,92]. The only exception in the first part of the study (the stage 1), was the level of IL-4, which is known as a pleiotropic anti-inflammatory cytokine that functions mainly by suppressing the pro-inflammatory environment. The highest values for IL-4 was observed in conditions imitating hypoglycemia, intermediate values in normoglycemia, and the lowest values in hyperglycemia. Being an important modulator of the immune system, IL-4 is primarily released by mast cells, Th2 cells, eosinophils, and basophils [93]. IL-4 can have either pro-inflammatory or anti-inflammatory impacts on astrocytes, with the outcome depending on the specific treatment and timing regimen [93,94]. It was reported that IL-4 modulates microglia homeostasis and may attenuate slowly progressing neurodegenerative disorders (e.g., amyotrophic lateral sclerosis). Our results may indicate that anti-inflammatory properties of IL-4 are most severely impaired in a hyperglycemic environment.

In 12 h after 0.2 μM LPS addition, a significant increase in the cytokine concentration was observed only for IL-6 and IL-8 (see Figure 5). It may be explained by the relatively low dose of LPS used in the study [95]. Thus, the 12 h period after LPS addition was probably too short to induce a significant rise of pro-inflammatory cytokine levels. Accordingly, in the subsequent measurements, made another 24 h after LPS addition, the increase in concentration could be observed for almost all cytokines (see Figure 6). This indicates that LPS did induce production of pro-inflammatory cytokines, although in the case of IL-1α, IL-1β, IL-4, and partially in case of IL-2 (hyperglycemic conditions), the time interval necessary for this effect was longer. The strong correlation between LPS administration and a rapid increase in the production of IL-6 and IL-8 has been reported in numerous studies performed to date [96–99].

Therefore, it appears that, at least in a controlled laboratory setting, LPS triggers the generation of various pro-inflammatory cytokines at varying time intervals. This aspect should be kept in mind when designing upcoming experiments using this BBB model. Additionally, it is worth noting that endothelial cells in the co-culture also exhibit reactions to LPS, and one element of future research may be to evaluate both the extent and timing of their inflammatory responses [100].

The role of the BBB in maintaining homeostasis is crucial, yet it also poses a significant obstacle for the drugs to reach the brain [101]. Developing drugs to address CNS disorders is exceptionally difficult, primarily because of their low bioavailability in the CNS due to the barrier function of the BBB [102].

The crucial part of the study (stages 3 and 4) was to verify if RSV—as a compound with documented anti-inflammatory properties—may cross the BBB and develop its anti-inflammatory action with the same efficacy at different glucose concentrations in MC. As shown in the previous in vivo studies using a different methodology, RSV's ability to cross the BBB may play a critical role in neuroprotection from the development of diseases linked to the inflammatory response in the CNS [103].

In one study, significant levels of RSV were found in the rodent's brain after intraperitoneal injection, reaching its peak at 4 h post-injection [104]. In order to clarify whether any correlation between human and rodent bioavailability exists during intraperitoneal administration of RSV, further intensive analysis is needed. Another in vivo study on RSV bioavailability in humans showed that RSV and its major metabolites were detected in plasma and CSF in patients after taking 500 mg of RSV orally once a day [105]. Despite several encouraging discoveries in human clinical trials, there are still numerous conflicting results, which can be partially attributed to variations in the dosing regimens applied [106].

In this in vitro study under conditions mimicking normoglycemia, only 0.61% of the RSV was detected in the BC, following its addition to the MC. This percentage was even lower under hypoglycemia- and hyperglycemia-mimicking conditions (0.53% and 0.55%, respectively). As documented by the cytokine profiles assessed in the BC, the potential of

RSV to counteract neuroinflammation in the context of normoglycemia is higher than in hyperglycemia- or hypoglycemia-induced BBB disruption.

Due to the inflammatory response and induced cell damage, conditions of hyper- and hypoglycemia are generally associated with increased BBB permeability compared to normoglycemia. We can speculate that because of RSV's effects on cell adhesion proteins, its neuroprotective activity may partially compensate the effect of dysglycemia. The specific mechanisms of RSV in maintaining or restoring BBB integrity might explain the observed decrease in RSV levels under hyper- and hypoglycemic conditions relative to normoglycemic conditions.

A future challenge would be to assess the uptake and the kinetics of RSV metabolism in the BC. This could help to determine whether RSV is being metabolized by endothelial cells and/or astrocytes (with this phenomenon increased in abnormal glycemic states in the MC) or if the low RSV recovery in the BC observed in this study is primarily due to limited penetration through the BBB.

It was reported that such a low bioavailability of RSV may reduce its anti-inflammatory properties [107]. Extensive metabolism of orally administered RSV in the liver and in the intestine results in an oral bioavailability lower than 1%. While in vitro studies demonstrate a significant beneficial biological effect of RSV within cells, it is reported that its distribution in tissues is exceedingly limited [108]. The insufficient absorption of RSV by human tissues constitutes an important challenge when it comes to applying fundamental scientific findings to clinical applications [106].

For this reason, numerous studies have aimed at enhancing RSV bioavailability, especially in the CNS. It has been found that RSV derivative (i.e., methylated) compounds can be more effective in crossing the BBB, both in vivo and in vitro, and might be a suitable path for the future research [109,110]. The bioavailability of RSV in the CNS could also be improved through encapsulation in nanoparticles. So far, this compound has been encapsulated within a variety of nanosystems, such as liposomes, lipid nanoparticles, and polymeric nanoparticles [111]. Furthermore, it has been demonstrated that the BBB penetrability of RSV is remarkably increased by its intrathecal administration [112]. This route bypasses the BBB to deliver the polyphenol directly to the CNS. It has been demonstrated to be effective in improving RSV bioavailability in the CNS. On the other hand, oral administration is the most common route for administering RSV due to its convenience and patient compliance. There is no doubt that optimization of the formulation composition and administration route is essential for improving RSV bioavailability and CNS penetration, ultimately enhancing its therapeutic potential in neuroinflammatory conditions.

In this study we analyzed six selected cytokines and not a whole group of more than 30 pro-inflammatory cytokines identified to date. Thus, it cannot be excluded that choosing some other pro-inflammatory cytokines could elicit different results [113].

It is crucial to emphasize that the outcomes we obtained are significantly affected by the specific experimental in vitro model we used. First and foremost, in our approach, the circulating blood in the vascular system was replaced with the EBM-2 medium. In this system, the lack of blood results in the elimination of variables influencing the interpretation of the results, including number of cytokines, cells (e.g., activated leukocytes, platelets), and hormones (e.g., insulin, sex steroids). Therefore, it is feasible to provide a simplified explanation of the results, which could be advantageous in highlighting the straightforward connections observed between glucose levels, LPS, RSV, and cytokine production in co-cultured endothelial cells in the MC and astrocytes in the BC. However, this approach leads to an incomplete model and reduces the possibility of directly applying these findings to real-world situations or using them in clinical studies.

Reproducing the results obtained from our in vitro study in animal models would be an essential step in validating the findings and understanding the broader implications. Animal models allow for the assessment of RSV's effects in a more holistic environment, considering interactions between various cell types, tissues, and organs within a living

organism. Additionally, animal models enable us to study the pharmacokinetics, bioavailability, and potential adverse effects of RSV.

Considering sex as a biological factor is also important, as sex differences can influence various physiological processes, including inflammation and glucose metabolism [114]. In animal models, both male and female subjects should be included to assess potential sex-specific differences in response to RSV treatment and under diabetic conditions. Differences in hormone levels, immune responses, and metabolic pathways between males and females may result in varying outcomes [115]. In control and diabetic animal models, it is possible that similar trends in RSV's anti-inflammatory effects may be observed in both males and females. However, sex-specific differences in response to RSV treatment and diabetic conditions could also be present, potentially affecting the magnitude or direction of the observed effects. Therefore, including both sexes in animal studies would provide a more comprehensive understanding of RSV's effects and their relevance across different populations.

Overall, while the in vitro study provides valuable preliminary data, further validation in animal models, considering sex as a biological factor, would be essential for advancing our understanding of RSV's potential therapeutic effects on brain inflammation and glucose metabolism.

Furthermore, diabetes, a multifaceted metabolic disorder, contrasts with artificially induced high blood sugar situations. Aberrations in the microvascular network of diabetic individuals, characterized by unusual blood vessel growth and heightened vascular density, could potentially affect the levels of cytokines and the activity of RSV.

The effects of multiple doses of glucose and RSV on cytokine production and inhibition, as well as RSV availability in the CNS, were not explicitly explored here. The study focused on investigating the anti-inflammatory effects of RSV on astrocyte cytokine profiles within an in vitro model of the BBB under varying glucose concentrations (2.2, 5.0, and 25.0 mmol/L). To optimize cytokine production inhibition and RSV availability in the CNS, future studies could explore different doses of both glucose and RSV. By systematically varying the concentrations of glucose and RSV, researchers could identify optimal conditions for minimizing cytokine production and maximizing RSV availability. This approach would provide insights into the dose–response relationships of glucose and RSV in modulating inflammation and could develop more effective therapeutic strategies for neurological disorders.

In conclusion, because the cytokine profile in the CNS is associated with glucose level fluctuations, we investigated if treatment with RSV is beneficial for diabetic patients with neuroinflammation. Supplementary treatment with this polyphenol in diabetic patients with neurodegenerative diseases may improve endothelial function, microvascular blood flow and metabolism, and—most importantly—reduce neuroinflammation. The obtained results suggest that the anti-inflammatory effects of RSV acting across the BBB are expected to be optimal in patients with adequate glucose control, ensuring normoglycemic conditions. For this reason, any therapeutic attempts with RSV in diabetes should be performed after or in parallel to restoring normoglycemia. Future in vivo studies in animals and human tests are needed, including those considering safety issues and side effects, especially excluding RSV toxicity. When establishing the ideal dosage for this substance, it is crucial to account for the biphasic dose–response of RSV in various human cell lines. This biphasic response entails positive effects at lower doses and detrimental effects at higher doses, making it a vital factor to consider. Implementation of the BBB in the in vitro model used in our study might be useful in future studies on RSV bioavailability, considering dose–response studies or using factors enhancing BBB penetration [116].

4. Materials and Methods

The study was performed between January 2021 and March 2022 in the Warsaw Medical University Center of Preclinical Research and Technology (CePT).

The study was conducted on commercially available human cell lines not requiring bioethical approval; nevertheless, we obtained approval from the Bioethics Committee from the Medical University of Warsaw on 14 December 2020 (No. AKBE220/2020).

The BBB model used in this study contained co-cultures of endothelial cells and astrocytes at passages 6–10. A detailed description of all procedures involved in the preparation of this in vitro model is provided elsewhere [117]. Endothelial cells and astrocytes were co-cultured, but they did not make direct contact due to a separation membrane (0.4 μm wide pores). Human cerebral microvascular endothelial cell line hCMEC/D3 was purchased from Cedarlane Cellutions Biosystems (Burlington, ON, Canada; catalogue # CLU512). This cell line is suitable for investigating human neurological pathways, as stated by the manufacturer. Endothelial cells were grown for 3 days to obtain 100% growth confluence in EBM-2 medium (Merck KGaA, Darmstadt, Germany; cat. # C-22211) supplemented with 5% Fetal Bovine Serum, 1% Chemically Defined Lipid Concentrate, 28.5 μM Ascorbic Acid, 62.5 μM bFGF- human Basic Fibroblast Growth Factor, 10 mM HEPES, 1.4 μM Hydrocortisone, and 100.000 U/L Penicillin-Streptomycin (all purchased from Thermo Fisher Scientific-Gibco™; Waltham, MA, USA). The cells proliferated in 5 special culture bottles, each having a surface of 75 square centimeters, provided by TPP, Techno Plastic Products AG (Trasadingen, Switzerland). Normal human astrocytes derived from healthy brain tissue were purchased from Thermo Fisher Scientific-Gibco™ (cat. # N7805100), together with Gibco™ Astrocyte Medium (cat. # A1261301), in which the cells were cultured for 4 days until they differentiated to glial fibrillary acidic protein (GFAP)-positive cells with a typical star-like morphology and reached 100% growth confluence in DMEM Gibco astrocyte medium, containing two additives: 1% N-2 Supplement and 10% One Shot Fetal Bovine Serum (FBS). The astrocytes were cultured in 5 special culture bottles, with 25 square centimeters of surface each, obtained from TPP Techno Plastic Products AG. After obtaining 100% confluence of both cell lines (astrocytes and endothelial cells), the cells were detached from the culture bottle and counted using a trypan blue dye. A mix of 10 μL of a cell suspension and 10 μL of trypan blue dye was placed in chamber's slide and subsequently in a Countess Automated Cell Counter (Thermo Fisher Scientific-Invitrogen). Each bottle contained about 1 million astrocytes per 1 mL of medium (5 million cells/flask) and about 2 millions of endothelial cells per 1 mL of medium (20 million cells/flask). Afterward, astrocytes were seeded into 24-well plates (Thermo Fisher Scientific-Gibco; catalog number A15690601). The following day, endothelial cells were introduced into insert wells, each with a see-through polyester (PET) membrane with a 0.4 μm pore size and a pore density of 2×10^6 cm^2. The culture surface area per individual well was 33.6 mm^2 (Greiner Bio-One GmbH—ThinCertTM, Frickenhausen, Germany; catalog number 662641). Nine 24-well plates were used in this study, with a total number of 216 wells. Each well was covered with Gibco Geltrex Matrix, a basement membrane extract with reduced growth factor properties, specifically employed for the adherence and long-term support of human cells. Astrocyte suspension (3×104 cells) was placed in each well and after 24 h the medium was exchanged. Afterwards, the inserts were coated with rat tail-derived type I collagen (Merck C7661; cat. # C7661) and placed in the wells. After the collagen had solidified to a gel-like state, a suspension of 6×10^4 endothelial cells was introduced into every individual insert. Following another 24 h period, the solution was replaced with a new one that had a pre-established glucose concentration. The obtained model of the BBB contained astrocytes representing the CNS side (the brain compartment, BC) and endothelial cells representing the circulatory side (the microvascular compartment, MC), as shown schematically in Figure 8. The cells were cultured in an incubator with controlled humidity at a temperature of 37 °C and a CO_2 concentration of 5%.

The integrity of the endothelial cell layer, corresponding to the integrity of the BBB model, was checked indirectly by assessing cell viability. At the stage of developing the BBB model, a correlation was shown between transendothelial electrical resistance (TEER) measurement values indicating loss of integrity and a reduction in cell vitality of more than 25% (compared to the average in a given assay). In the control (reference) group, the

average TEER results obtained at 2 h intervals were for periods 1–12, 13–24, and 25–36 h, respectively: 140.4 ± 11, 121.2 ± 09, and 111.6 ± 10 ($\Omega.cm^2$ ± SEM). The decrease in endothelial cell vitality above 25% correlated with a decrease in the TEER value by at least 30%, compared to the reference group, on average by 30.03 ± 4.7, 31.4 ± 4.5, and 33.6 ± 5.2 (% SEM) for the periods 1–12, 13–24, and 25–36 h, respectively.

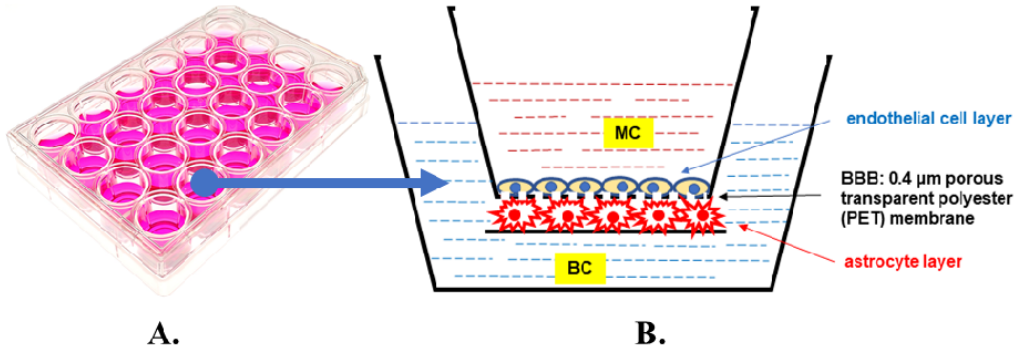

Figure 8. Multi-well plate used for co-culture of endothelial cells and astrocytes (**A**) and the schematic cross-section of the single well illustrating the blood–brain barrier (BBB) model used in this study (**B**). For a detailed description, see Section 4.

The basis for adopting a specific time frame for conducting the experiment, including the intervals for administering specific doses of the substances used, was data, both our own and in the literature, regarding the dynamics of changes in cytokine profiles during the LPS-induced inflammatory reaction and the speed of manifestation of the anti-inflammatory effect of RSV in in vitro culture conditions. On the other hand, the duration of the experiment took into account the possibility of maintaining the stability (integrity) of the BBB model we used.

The three study groups were established, differing in glucose concentrations in the MC. The culture media administered into the MC within the wells of group I, group II, and group III contained D-glucose concentrations mimicking hypo-, normo-, and hyperglycemia (40 [2.2], 90 [5.0], and 450 [25.0] mg/dL [mmol/L], respectively). To verify if glucose concentration itself affects inflammatory response, various cytokine levels in the BC were measured 24 h after glucose addition (stage 1 of the study). For each cytokine, six independent samples were analyzed.

The following inflammatory cytokine levels were measured: IL-1 α, IL-1 β, IL- 2, IL-4. IL-6, IL-8, with the Multi-Analyte ELISArray Kit (Qiagen-Q4Lab, Hilden, Germany; cat. # 336161), according to the instructions of the manufacturer. A multi-well ELISA microplate, covered with a variety of antibodies tailored to specific targets, enabled the capture of diverse cytokines. Every ELISArray microplate carried biological samples, in addition to positive and negative control samples. The ELISA test was carried out strictly according to the manufacturer's protocol. The absorbance was determined at a 450 nm and 570 nm wavelength. Subsequently, the recorded values at 570 nm were subtracted from those at 450 nm when wavelength correction was possible. This measurement was conducted using a monochromator-based microplate reader, the Biochrom Asys UVM 340. This device, manufactured by Biochrom-Harvard Bioscience in Holliston, MA, USA, has a wavelength range of 340–800 nm and can measure optical density (OD) in the range of 0–3.2. The manufacturer specifies the reproducibility of this instrument as 0.8%, with an accuracy of 0.5% from 0.1 to 1.0 OD at 450 nm and 1.0% from 1.0 to 2.0 OD at 450 nm, with deviations of 0.005 OD and 0.010 OD, respectively. Six repetitions of each measurement were conducted, and subsequently, the mean values in absorbance units (Au) were determined for the

specified cytokine concentration, with the results expressed as pg/mL ± SD (pg/mL ± SD). The negative control samples, which were in the form of Sample Dilution Buffer, were placed in the initial row of the microplate, denoted as row A. The positive control samples, consisting of the Antigen Standard Cocktail, were positioned in the final row, specifically designated as row H.

In this study, a solution of LPS with a final concentration of 0.2 µM was introduced to the culture plates forming the BBB on the side of the BC to create conditions resembling neuroinflammation. After 12 and 36 h, the ELISA was used to examine the cytokine profiles, including IL-1 α, IL-1 β, IL-2, IL-4, IL-6, and IL-8, in various study groups (labeled as groups I–III). These groups had different levels of glucose in the culture medium. Each measurement of cytokine levels represented the average value derived from six determinations in the experimental samples obtained from the brain cells. These activities constituted the **2nd phase of the study**.

A study to investigate how LPS treatment affects the shape of astrocytic cells cultured under different glucose conditions was conducted. After a 36 h culture period in a model simulating the BBB, three study groups were established, with some receiving LPS and others not. These cells were fixed in a 3% paraformaldehyde (PFA) solution in PBS for 30 min at room temperature. Subsequently, the cells were embedded in paraffin and stained with hematoxylin and eosin (H&E).

To examine the cells, a Zeiss Primovert inverted cell culture microscope was used, equipped with various light sources, including HAL 35 W and a 3W LED with infinity optics, along with a Zeiss Axiocam 105 Color camera for image capture. The acquired images using ZEN 2.3 software were analyzed.

Furthermore, the paraffin-embedded sections under a light microscope (Leica DM 400B) with the expertise of two experienced neuropathologists were examined. Each group was assessed using 50 images. High-resolution photographs of the H&E-stained astrocytes were taken with a camera attached to the microscope. The purpose of this preliminary assessment was to determine whether there was a valid reason for employing computer image analysis procedures.

To investigate how RSV affects the inflammatory response 36 h after introducing LPS, RSV solution with a final concentration of 50 µM was applied to each sample in the MC. Following this, an immunoenzymatic assay, specifically the Multi-Analyte ELISArray Kit, was employed to quantify the levels of cytokines such as IL-1 α, IL-1 β, IL-2, IL-4, IL-6, and IL-8 after a 24 h interval, signifying the conclusion of **the 3rd stage of the study**. Each measurement was performed six times, and subsequently, the average values were subjected to statistical analysis.

Finally (**stage 4 of the study**), transfer of RSV between the compartments was measured by the comparison of RSV concentration in the BC between the studied hypo-, normo-, and hyperglycemic groups (groups I-III) 12 and 24 h after administration of LPS. The ELISA kit (MyBioSource, Inc., San Diego, CA, USA; cat. # MBS2700660) was used. All of the measurements were performed six times, and the mean values were statistically analyzed.

To ensure the research model (BBB) was validated and the results could be correctly understood, suitable control groups were designed for each phase of the study, as outlined in Table 1.

Table 1. The set of controls: content of culture media used at various stages of the study in control groups from the microvascular compartment (MC) side of the in vitro model of the blood–brain barrier.

Control Groups	Basal Medium	Glucose Medium	LPS Solution	RSV Solution
1st stage of the study	+	–	–	–
2nd stage of the study	+	–	+	–
3rd stage of the study	+	–	+	+

In the initial phase of the study, a control group was established using a basal medium devoid of any glucose concentration.

During the subsequent stage of the study, which involved the addition of LPS, another control group was formed. This control group utilized a basal medium with 0.2 μM LPS but excluded any glucose solution.

In the final phase of the study, a control group was designed with a basal medium containing 0.2 μM LPS and 50 μM RSV but devoid of glucose. Each control group underwent measurements six times to ensure robust data collection and analysis.

After obtaining the results and verifying the completeness of the data, the normal distribution of the results in all study groups was verified. We used the R programming language and its inherent capabilities (The R Project for Statistical Computing; version 4.0.5) for statistical analysis, in addition to employing the Mann–Whitney U test. The results are presented as the average values with their corresponding standard deviations (SD), and statistical significance was determined if the *p*-value was lower than 0.05.

5. Conclusions

In our study, RSV exhibited anti-inflammatory action by decreasing the concentration of all of the pro-inflammatory cytokines (with significant decreases noted for most of them) in all three study groups, corresponding to different levels of systemic glycemia. Regardless of the co-existing glucose concentration in the MC, the cytokine concentrations in the BC were lower in RSV-administered samples compared to RSV-free samples. Importantly, the largest decrease was observed in normoglycemia-simulating conditions (group II) for IL-1β, IL-6, and IL-8 ($p < 0.05$). It suggests that normoglycemia provides an optimal environment for anti-inflammatory action of polyphenols such as RSV. The cytokine concentrations in the normoglycemic environment were decreased to various extents, with the most pronounced effect on IL-1β, but also with great impact on IL-8. The hitherto obtained results suggest a correlation between RSV efficacy and normal glucose concentration in MC for most analyzed cytokines, although the concentration of the only anti-inflammatory cytokine, IL-4, was the most affected by RSV in hypoglycemia conditions.

Thus, referring to the anti-inflammatory effects of RSV at the level of the CNS, the importance of adequate glycemic control and restoring normoglycemia in diabetic patients with diseases and conditions such as Alzheimer's disease, Parkinson's disease, stroke, multiple sclerosis, and traumatic brain injury should be appreciated. Anti-inflammatory agents such as RSV could most effectively perform their functions in the normal range of glucose concentration, which results, among others, in the optimal neuroprotection and reduction in neuroinflammation. Further in vivo studies on this flavonoid are worth conducting to evaluate its neuroinflammation-preventing effect, especially in patients with diabetes.

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Institutional Review Board Statement: The study was conducted on commercially available human cell lines not requiring the bioethical approval; nevertheless, we obtained approval from the Bioethics Committee from the Medical University of Warsaw on 14 December 2020 (No. AKBE220/2020).

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Abbreviations

AGEs	advanced end glycation products
ALP	alkaline phosphatase
AMPK	AMP-activated protein kinase
BBB	blood–brain barrier
BC	brain compartment
CNS	central nervous system
CSF	cerebrospinal fluid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	extracellular signal-regulated kinase ½
ETC	electron transport chain
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	hematoxylin and eosin
IFN-γ	interferon gamma
IGT	impaired glucose tolerance
ILs	interleukins, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-17A
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MC	microvascular compartment
NAD	nicotinamide adenine dinucleotide
NAD^+	oxidized form of NAD
NAMPT (or NAmPRTase)	nicotinamide phosphoribosyl transferase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
P38-MAPK	mitogen-p38-activated protein kinases
PBS	phosphate-buffered saline
PET	polyester
PFA	paraformaldehyde
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator 1α
PI3K/Akt	phosphoinositide 3-kinase/protein kinase B
PRR	pattern recognizing receptor
PTS	protein transport system
ROS	reactive oxygen species
RSV	resveratrol
SIRT, SIRT1	sirtuin, sirtuin 1
T2D	type 2 diabetes
TEER	transendothelial electrical resistance
TNF-α	tumor necrosis factor alpha
γ-GTP	gamma-glutamyltranspeptidase

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7. PODSUMOWANIE I WNIOSKI

7.1. Podsumowanie

W **Tabeli 1.** przedstawiono wyniki uzyskane na poszczególnych etapach badania, podsumowujące uzyskane stężenia cytokin i RSV w trzech grupach badanych.

	Hipoglikemia	**Normoglikemia**	**Hiperglikemia**	**Główne ustalenia**
Średnie stężenie cytokin w BC (pg/ml) po 24 h inkubacji komórek w różnych stężeniach glukozy w MC	Wyższe stężenie: IL-1a, IL-6, IL-8, GM-CSF, IL-17A, IL-12 w porównaniu do grupy II ($p<0.05$).		Wyższe stężenie: IL-1a, IL-6, IL-8, GM-CSF, TNF-a, INF-g, IL-12 w porównaniu do grupy II ($p<0.05$).	Stan zapalny może być wywołany zarówno przez hipo-, jak i hiperglikemię.
Średnie stężenie cytokin w BC (pg/ml) 12 h po wprowadzeniu roztworu LPS do BC w porównaniu do stężenia cytokin bez roztworu LPS.	Wzrost stężenia IL-2, IL-6 i IL-8 ($p<0.05$).	Wzrost stężenia IL-2, IL-6 i IL-8 ($p<0.05$).	Wzrost stężenia IL-6 i IL-8 ($p<0.05$).	Oczekiwany efekt prozapalny LPS nie wystąpił po 12 h po jego podaniu.
Średnie stężenie cytokin w BC (pg/ml) 36 h po wprowadzeniu roztworu LPS do BC w porównaniu do stężenia cytokin 12 h od wprowadzenia roztworu LPS	Wzrost stężenia: [IL-1b, IL-2, IL-4, IL-6, IL-8, IL-12, INF-g, TNF-a ($p<0.05$)], [GM-CSF, IL-17A ($p<0.02$)].	Wzrost stężenia: [IL-1a, IL-1b, IL-4, IL-6, IL-8, IL-12, INF-g, TNF-a ($p<0.05$)], [GM-CSF, IL-17A ($p<0.02$)].	Wzrost stężenia: IL-1a, IL-1b, IL-2, IL-6, IL-8, [IL-12, INF-g, TNF-a, GM-CSF ($p<0.05$)], [IL-17A ($p<0.02$)].	Efekt prozapalny LPS został zaobserwowany po 36 h od jego podania.

Średnie stężenie cytokin w BC (pg/ml), 36 h po wprowadzeniu roztworu LPS do BC oraz 24 h po wprowadzeniu roztworu RSV do MC	Spadek stężenia: IL-1b, IL-2, IL-4, IL-6, IL-8, IL-12, IL-17A, IFN-y, GM-CSF, TNF-a ($p<0.05$).	Spadek stężenia: IL-1b, IL-2, IL-4, IL-6, IL-8, IL-12, IL-17A, IFN-y, GM-CSF, TNF-a ($p<0.05$), bardziej wyraźny spadek cytokin w porównaniu do pozostałych grup.	Spadek stężenia: IL-1b, IL-2, IL-4, IL-6, IL-8, IL-12, IL-17A, IFN-y, GM-CSF, TNF-a ($p<0.05$).	Warunki normoglikemii stanowią optymalne środowisko dla działań przeciwzapalnych RSV.
Średnie stężenie RSV w BC (ng/ml) po 12 h i 24 h od wprowadzenia roztworu RSV do MC	Niższe stężenie RSV w porównaniu ze stężeniem RSV w grupie II ($p<0.02$).	Najwyższe stężenie RSV w porównaniu ze stężeniami RSV w grupach I i III ($p<0.02$).	Niższe stężenie RSV w porównaniu ze stężeniem RSV w grupie II ($p<0.02$).	Hipo- i hiperglikemia zmniejszają przepuszczalność BKM dla RSV.

BKM – bariera krew-mózg; BC – brain compartment; LPS – lipopolisacharyd; MC – microvascular compartment; RSV – resweratrol.

Tabela 1. Analiza zmian w stężeniu cytokin oraz porównanie stężenia RSV w trzech grupach badanych, odpowiadających stanom hipo-, normo- i hiperglikemii.

Nieprawidłowe stężenie glukozy w MC indukuje odpowiedź zapalną w BC, wyrażone w postaci wzrostu stężenia cytokin prozapalnych ($p<0.05$). Zarówno warunki hipo-, jak i hiperglikemii prowadzą do rozwoju zapalenia, co jest zgodne z wynikami uzyskanymi przez innych badaczy [53,54]. Po 12 h od wprowadzenia roztworu LPS do BC nie uzyskano oczekiwanego wzrostu stężenia większości badanych cytokin, z wyjątkiem IL-2, IL-6 i IL-8. W opublikowanych badaniach niezależnych autorów, także wykazano korelację pomiędzy podaniem roztworu LPS i szybkim wzrostem poziomu cytokin IL-6, IL-8 [55,56]. Oczekiwaną (przewidywaną) odpowiedź zapalną, w postaci wzrostu stężenia badanych cytokin, zaobserwowano po 36 h od wprowadzenia roztworu LPS do przestrzeni

BC ($p<0.05$). LPS indukuje odpowiedź zapalną w zróżnicowanych interwałach czasowych, co należy wziąć pod uwagę, planując przyszłe badania *in vivo*.

Po 36 h hodowli komórek po podaniu roztworu LPS, analizując losowo wybrane obrazy, nie zaobserwowano istotnych zmian w morfologii ani w żywotności astrocytów (ocena morfologii komórek była uzupełniona barwieniem przyżyciowym astrocytów w teście wykluczenia z użyciem błękitu trypanu). Po dodatkowej konsultacji z ekspertami w dziedzinie histopatologii, zrezygnowano z dalszych analiz morfometrycznych.

Po 24 h od wprowadzenia roztworu RSV do MC zaobserwowano obniżenie stężenia cytokin (IL-1β, IL-2, IL4, IL-6, IL-8, IL-12, IL-17A, IFN-γ, TNF- α, GM-CSF) w BC we wszystkich grupach badanych ($p < 0.05$). Jednakże najbardziej wyraźny spadek cytokin (IL-1β, IL-2, IL-6, IL-8, IL-12 IL-17A, IFN-γ, TNF-α, GM-CSF) zanotowano w grupie II, imitującej normoglikemię, co sugeruje, iż warunki normoglikemii stanowią optymalne środowisko dla działania przeciwzapalnego RSV. Po wprowadzeniu roztworu RSV do MC, uzyskano wyższe stężenie RSV w BC w grupie II w porównaniu do pozostałych grup, imitujących warunki hipo- i hiperglikemii. Ze względu na reakcję zapalną i spowodowane nią zmienione właściwości komórek, warunki hipoglikemii i hiperglikemii generalnie wiążą się ze zwiększoną przepuszczalnością BKM w porównaniu z normoglikemią [57,58]. Można zakładać, że ze względu na wpływ RSV na białka adhezyjne komórek, jego działanie neuroprotekcyjne częściowo kompensuje efekt dysglikemii. Wyniki niektórych badań sugerują, że RSV wykazuje wpływ na integralność i funkcję BKM, co pozwala regulować przenikanie substancji do OUN, w tym leków, tym samym normalizować zaburzenia w OUN [59,60]. Efektywne mechanizmy działania RSV w kierunku utrzymania lub przywracania integralności BKM mogą tłumaczyć obserwowane niższe stężenia RSV w warunkach hipo- i hiperglikemii w porównaniu z poziomem RSV w warunkach normoglikemii.

Pomimo słabej penetracji RSV do BC, korzystny efekt działania RSV (w postaci obniżenia stężenia cytokin prozapalnych) mógłby zostać wykorzystany w dalszych badaniach, mających na celu ograniczenie odpowiedzi zapalnej w OUN u pacjentów z chorobami neurodegeneracyjnymi i przebiegającymi z zaburzeniami regulacji poziomu glukozy we krwi.

7.2. Wnioski

– Profil cytokin prozapalnych w OUN zależy od poziomu glukozy w przestrzeni odpowiadającej naczyniom mikrokrążenia mózgowego (MC). W porównaniu z normoglikemią, zarówno hipo- jak i hiperglikemię cechuje podwyższenie stężeń badanych cytokin w BC.

– W warunkach *in vitro* z użyciem modelu BKM, normoglikemii towarzyszy największy wzrost przepuszczalności BKM dla RSV, co sprzyja jego penetracji do przestrzeni mózgowej (BC). Konieczne są dalsze badania, niezbędne do zrozumienia konkretnych efektów wpływu stężenia glukozy na penetrację RSV do OUN. Czynniki takie jak odpowiednia dawka RSV, forma i czas podania, zmienności indywidualne, będą miały z pewnością znaczenie w warunkach klinicznych.

– RSV wykazuje działanie przeciwzapalne, które przejawia się obniżeniem poziomu cytokin w obrębie BC we wszystkich badanych grupach, przy czym do najwyższego spadku stężenia cytokin dochodzi w warunkach normoglikemii. Dlatego można przyjąć, że środowisko normoglikemii jest optymalne dla działań polifenoli przeciwzapalnych, takich jak RSV.

– Zastosowanie terapeutyczne RSV może być potencjalnie korzystne u pacjentów z chorobami związanymi z neurozapaleniem oraz cukrzycą, gdyż ogranicza nasilenie odpowiedzi zapalnej w obrębie OUN. Należy rozpocząć/kontynuować dalsze intensywne badania przedkliniczne z wykorzystaniem modeli zwierzęcych, a następnie kliniczne, aby zweryfikować tę tezę z badania na modelu pozaustrojowym BKM.

– Ponieważ w przypadku większości badanych cytokin, prawidłowe stężenie glukozy w MC koreluje z wyższą skutecznością i dłuższym okresem działania RSV, wszelkie próby terapeutyczne z użyciem RSV u pacjentów z cukrzycą i chorobami OUN przebiegającymi z neurozapaleniem (np. choroba Alzheimera, choroba Parkinsona, SM, chorzy po udarze mózgu) powinny być przeprowadzane po przywróceniu lub równolegle do przywracania normoglikemii.

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9. OPINIA KOMISJI BIOETYCZNEJ

Komisja Bioetyczna
przy Warszawskim Uniwersytecie Medycznym

Tel.: 022/ 57 - 20 -303
Fax: 022/ 57 - 20 -165

ul. Żwirki i Wigury nr 61
02-091 Warszawa

e-mail: komisja.bioetyczna@wum.edu.pl
www.komisja-bioetyczna.wum.edu.pl

Warszawa, dnia 14 grudnia 2020r.

AKBE/ 220 / 2020

Lek. Justyna Komorowska
Katedra i Zakład Patologii Ogólnej i Doświadczalnej
ul. Pawińskiego 3c
02-106 Warszawa

OŚWIADCZENIE

Niniejszym oświadczam, że Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym w dniu 14 grudnia 2020 r. przyjęła do wiadomości informację na temat badania pt.:"Wpływ stężenia glukozy na poziom bariery krew-płyn mózgowo-rdzeniowy na właściwości przeciwzapalne resweratrolu w obrębie komórek śródbłonkowych i astrocytów-badanie na modelu in-vitro." Przedstawione badanie nie stanowi eksperymentu medycznego w rozumieniu art. 21 ust. 1 ustawy z dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty(Dz.U. z 2018 r. poz. 617) i nie wymaga uzyskania opinii Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym, o której mowa w art. 29 ust. 1 ww. ustawy.

Przewodnicząca Komisji Bioetycznej

Prof. dr hab. n. med. Magdalena Kuźma –Kozakiewicz

PROF. DARIUSZ SZUKIEWICZ

10. OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI

Warszawa, dn. 24.05.2024 r.

prof. dr hab. n. med. Dariusz Szukiewicz

OŚWIADCZENIE

Jako współautor pracy pt. „Review of beneficial effects of resveratrol in neurodegenerative diseases such as Alzheimer's disease" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór, udzielanie merytorycznych uwag, ocena całości pracy, zatwierdzenie ostatecznej wersji artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Justyny Komorowskiej w powstawanie publikacji określam jako 90 %,

obejmował on: wybór tematu, przegląd literatury, ocenę jakości i wiarygodności zebranych materiałów, porównywanie wyników różnych badań, analizę i interpretację danych, syntezę informacji, cytowanie źródeł, pisanie tekstu, przygotowanie ilustracji, tabel oraz bibliografii.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Justyny Komorowskiej.

.....

(podpis oświadczającego)

Warszawa, dn. 24.05.2024 r.

dr n. med. Mateusz Wątroba

OŚWIADCZENIE

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M. Wątroba

(podpis oświadczającego)

Warszawa, dn. 24.05.2024 r.

prof. dr hab. n. med. Dariusz Szukiewicz

OŚWIADCZENIE

Jako współautor pracy pt. „The Role of Glucose Concentration and Resveratrol in Modulating Neuroinflammatory Cytokines: Insights from an In Vitro Blood-Brain Barrier Model" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: pomoc w opracowaniu projektu badawczego, nadzór nad pracą badawczą, drobne poprawki przed wysłaniem pracy do druku.

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(podpis oświadczającego)

Warszawa, dn. 24.05.2024 r.

dr hab. n. med. Anna Henriques dos Santos de Sepulveda

OŚWIADCZENIE

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(podpis oświadczającego)

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M. Wątroba

(podpis oświadczającego)

Warszawa, dn. 24.05.2024 r.

Małgorzata Bednarzak

OŚWIADCZENIE

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Małgorzata Bednarzak

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OŚWIADCZENIE

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Jako współautor pracy pt. „Anti-Inflammatory Action of Resveratrol in the Central Nervous System in Relation to Glucose Concentration—An In Vitro Study on a Blood–Brain Barrier Model" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udzielanie merytorycznych uwag w trakcie pisania pracy, recenzowanie oraz wnoszenie poprawek do tekstu.
Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Justyny Komorowskiej w powstawanie publikacji określam jako 80 %, obejmował on: pomysł i projekt badania, sformułowanie celów badawczych, zbieranie i analizę danych, przeprowadzenie analizy statystycznej, interpretację wyników, pisanie tekstu artykułu, przegląd literatury, przygotowanie ilustracji i tabel, poprawki tekstu zgodnie z wymaganiami czasopisma naukowego.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Justyny Komorowskiej.

ASepulveda

(podpis oświadczającego)