Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 3163 – 3174
Received 05 March 2021; Accepted 01 April 2021.

Myricetin and Berberine Combination Ameliorates Brain Damage Induces by Cerebral Ischemia/Reperfusion Injury in Rats

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Summary:

Background: Cerebral ischemic/reperfusion (I/R)injurymayresultin considerable tissuedamageviainductionofinflammatorymediatorsandoxidativestressinbrain tissue.Theaimofthepresentstudy wastoinvestigatevalueofusingcombinedmyricetin plus berberinetreatmentinprotection againstshort-term globalcerebralI/Rinjuryin rats. **MaterialandMethods:**Thirty sixWistar-albinoratswereenrolledinthestudy,divided intofourgroupsincluding Shamgroup,I/Rgroup,I/R+(control-vehicle DMSO)andI/R+ myricetin 50 mg/kgplus berberine5 mg/kginjectedintraperitoneally1 hourbefore inductionof ischemia. Measurementof braintissueIL-1β,ICAM- 1, caspase-3, Notch 1 andJagged1wasdoneafteronehourofreperfusioninadditiontotheassessmentof brain infarcted areaand histopathologicaltissue analysis.

Results: Myricetinandberberinecombinationattenuatesthecerebral I/Rinjury induced increase in inflammatory cytokine (IL-1β), adhesion molecule (ICAM-1) and proapoptotic enzyme (caspase-3).Additionally,itreducesthe size of infarcted area and histopathologicaldamage.However,suchprotectiveeffectwasnotfoundtobe mediated by Notch1signalingpathwaybecausedrugstreatmentdidn't showanychangesinthe increased levels of Notch1 and Jagged 1 seen in brain withI/Rinjury.

Conclusions: Myricetinandberberine combinationhasa neurocytoprotectiveoutcome againstcerebralI/Rinjurywhichismanifestedasanti-inflammatory anti-apoptoticeffect thatpreservedcellstructure andviability,neverthelessthiseffectisnotmediatedthrough Notch 1 signalingpathway.

Keyword:CerebralI/R,Myricetin,IL-1β,Notch 1,Jagged

Introduction:

Ischemic stroke ismorecommonthan hemorrhagic andabout87% of reported cases fall underthiscategory andover80%ofcardiovasculardeathsoccurinlow-andmiddle-incomecountries**(1)**.Limitationofbloodflowthroughthecerebralartery dueto thrombosis can cause ischemic stroke which affect cellular homeostasis due to insufficientoxygenand nutrientsupply**(2)**.Reperfusioninjuryisdefinedasfurther deterioration of ischemic brain tissue by a series of biochemical events upon reestablishingcerebralcirculation**(3)**.Asinotherorgansystems,aperplexingsequence of events can lead tocerebral damage at thesiteofischemiareperfusion. Cellular acidosis causesimpairmentinenzymaticactivitiesandchromatinclumping insidethenucleus,the

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reestablishmentofbloodflowtoischemictissuedeliversoxygenleading toexcessive increase inreactiveoxygenspeciesproductionbecause of thelower levelof antioxidative agentsinthecellculminatingendothelialdysfunctionandDNAdamage,inaddition many inflammatory cascades are triggered by oxidative stress leading to massive cytokinesproductionlikeIL-1βandICAM-1whichwilleventually resultincelldeath due todestructionof numerouscellular structures**(4)**. While inflammationistheessential processinthepathophysiology ofI/Rinjury,leukocytesinfiltrationisusually thestarting event.Intheperiodof reperfusion,chemotacticagentscausetheadherence ofactivated leukocytes toendothelialcells. Matrixmetalloproteinasesand neutrophil derived oxidants are thenproducedwhichcausedamage tothebloodbrainbarrier,theleukocytesthen leavethecapillary and penetratethebraintissuetoreleaseinflammatorymediatorswhich damage the ischemic penumbra **(5)**. Studies have shown that reducing ICAM-1 expressiondue todecreasingIL-1βproductionwasassociatedwithdecreasedinfarction size**(6)**.Apoptosisisactivatedinresponsetohypoxicischemicinsultand inresponseto oxidativestressinreperfusioninjury,theischemicpenumbraiscommonly themost affectedareaby apoptosis**(7)**.Caspase-3isactivatedby bothintrinsicandextrinsic pathways.Oncecaspase-3isactivated,itwillcleavenumerousproteinsubstrates,this willeitherleadtotheactivationofcertainproteinsthatenhancecellkilling orinactivate otherproteinsthatareimportantforcellsurvivalwhichwilleventuallyenhancecell killing**(8)**.Notch1signaling isvery essentialfornervoussystemdevelopment,itis expressedsignificantly inneuralstemcellsandneuroblastsinwhichitcontrols proliferation,differentiationandmaintenance innormalor pathogenic state,thismakesit crucialfor neurogenesisandneuralspecification**(9)**.Thedetrimentaleffectof Notch signalingpathwayactivationincerebralischemiawasillustratedby studieswhichhave shownthatNotchmay haveasignificantpartinactivationofmicrogliaandlymphocyte infiltrationincerebralischemia**(10)**. Jagged1(JAG1)isoneofthe5cellsurfaceligandsthatoperatemainlyonthepreservedNotchsignalingpathway.Jagged 1hasbeenshown tobe upregulatedincerebralischemia asanadaptive responsetoenhanceneurogenesisin responsetostroke**(11)**.Increasingconsiderationinthefieldofdrugindustry hasbeen centeredonthe neuroprotective effectsof naturalcompoundsfromtraditionalherbal medicine,herbalcompoundswithanti-oxidative,anti-inflammatory oranti-apoptotic propertiesshowedprotectiveortherapeuticeffectsinmany animalmodelsofcerebral ischemia**(12)**. Myricetinisaphenoliccompoundcommonly foundintea,berries, vegetablesand redwine.Itexistseither asafreeformorasaglycoside**(13)**.Myricetin demonstratedbeneficialeffectsinmany animalmodelsofI/Rinjury.InintestinalI/R injury,itwasfoundtodecreaseproductionofinflammatory cytokineslikeTNF-α,IL-1β, andIL-6in addition to reducingMDAlevel and increasingthelevels of SOD and GSH in theintestinaltissuesleading toimprovementinI/R-inducedratintestinalinjury**(14)**. Berberine isa naturalalkaloidof theisoquinoline class.Variousstudieshavebeen conductedonberberineoverthelasttwodecadesrevealingmany beneficial pharmacologicalandtherapeutic effectsfor this alkaloid**(15)**.The mainobjective of our study istoassessthepotentialneuroprotectiveeffectofmyricetininaglobalmodelof cerebralischemiareperfusioninjury

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afterbilateralcommoncarotidartery occlusion (BCCAO)inrat,andtoinvestigatethepotentialroleofNotchsignaling pathway in mediatingtheseeffects.

Materials andMethods:

Animalsandexperimentalprocedures: Atotalof36adultmale WistarAlbinorats weighing(200-400g)werepurchasedfromCollegeofScience–UniversityofZakho. They keptatUniversity ofKufafortwo weeksforadaptation,thenratswere distributed randomly into4groupsasfollows:**Group1(Shamgroup):**Inthisgroupofrats,the anestheticandsurgicalprocesseswere performedwithoutBCCAO.**Group2(Control group):**Inthisgroupofrats,BCCAO wasperformedfor 30min.,andthenreperfusion wasallowedfor1hour.**Group3(Control-Vehiclegroup):**Onehour before ischemia, theratswereinjectedintraperitoneally bydimethylsulfoxide(DMSO)andthenBCCAO wasperformedfor30min.,followedby reperfusionfor1hour.**Group4(Treatment group):**Inthisgroup ofrats,myricetin50mg/kgplusberberine 5mg/kg wasinjected intraperitoneally 1 hour before BCCAO **(16)**. The experiment was approved by University of Kufa-Animal Care and Research Committee, and the investigation accordingtotheLaboratoryAnimalsGuideCare.**Inductionofglobalbrainischemia:** Aglobalmodelofbrainischemiawasinducedby BCCAO**(17)**,animalstemperaturewas keptatabout37°Cbytheaidofalightbulb,andtheratswereanesthetizedby ketamine atadoseof100mg/kgandxylazineatadoseof10mg/kgintraperitoneally**(18)**.Thenafterbeingplacedonthebackandfix edfirmlyinthesupineposition,asmallincision wasperformedinthemiddleoftheneckandthecarotidarterieswere isolatedfromthe vagalnervesbilaterallyandoccludedbyminivascularclampstoinduceischemia,after 30minutesofocclusiontheclampswereremovedandreperfusionwasallowedfor1 hour.

Preparationofsamples: Afteronehourofreperfusion,theratsweredecapitated,and thebrainswereisolatedasinandwashedinice-coldPBS,they werekeptoniceand weighedthensectionedinto3maincoronalslices,one slice waskeptin10% formalinfor histopathologicalanalysis.The other slicewaskeptinthefreezerat-20for20minutesto enablefurther sectioningtomore uniformcoronalslicesfor TTCstaining,while thelast slicewasmixedin1:10 (w/v)ratiowithicecold 0.1MPBS(H7.4)whichcontains1X cocktailproteaseinhibitor,and0.2%tritonX-100thenhomogenizedby ultrasonicliquid processor,thehomogenateswerethencentrifugedat15,000g for30minutesat4°Cand thesupernatantswere withdrawnandstoredat-80°Cfor measurementof other markers byELISA technique **(19).**

Measurement ofstudyparameters:

MeasurementoftissueIL-1β,ICAM-1andcaspase-3: Theymeasured byElabscience ratELISAkitswhicharesandwich typeenzyme–linkedimmunosorbentassay according to themanufacturer's protocol.

Measurement of tissue Notch 1: Notch 1wasmeasured byRayBio®ratNotch-1 ELISAkitwhichquantitatively measuresNotch1receptorinplasma,serumandtissue homogenates accordingto the manufacturer's protocol.

MeasurementoftissueJagged1: Jagged1wasmeasuredbyRayBio®ratJagged1

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ELISAkitwhichquantitatively measuresjagged1proteininplasma,serumandtissue homogenates accordingto the manufacturer's protocol.

TTCstainingandmeasurementofinfarctionarea:Estimationof theinfarction area wasaccomplishedby 2,3,5–triphenyltetrazoliumchloride(TTC)stainingtechnique**(20)**, thetetrazoliumsaltisreducedbydehydrogenaseswhicharepresentinthemitochondria toaredcoloredformazanproductsoviabletissuewillbestainedredwhileinfarcted tissuewillbeleftunstained.TTC0.2% (w/v)stainsolutionwasfreshly preparedby dissolving theTTCpowderinPBS,thestaining procedure wasasfollows:2mmthick coronalsliceswasquickly sectionedafterbrainisolation,theslices were keptmoistened incoldPBSduring theslicing process,thentheslicesweretransferredtothefreshly prepared 0.2%TTC solution in a flat bottomed covered dish and incubatedfor30 minutes at37°Cindarksincethestainislightsensitive,thedishwasshakenevery5minutes, thenthestainsolutionwasremovedandthesliceswerewashedwithPBS,finallythesliceswere keptin 4% bufferedformalinina flatbottomedtransparentdishand photographed.Thephotoswereanalyzedusing(ImageJ)software,thestainlessareas were defined as infarcted and calculated, and then the infarction percentage was calculatedand comparedbetween different treatment groupsand the control group.

Tissuesamplingforhistopathology: The formalinfixedslicesunderwenttissue processing tobeembeddedinparaffinwaxand thenwerelongitudinally cutinto5µm sections,thesectionswerethenstainedwithHandEstainfor histopathological examination**(21)**.

Histopathologicalanalysisandscoringofcerebralinjury: The histopathological analysis for the scoringof brain damagewas determined as follows **(22)**:

0, (normal) no morphological signs of damage;

1, (slight) edema oreosinophilic ordark (pyknotic) neurons or dark shrunkcerebral purkenje cells;

2, (moderate) at least twosmall hemorrhages;

3, (severe) clearlyinfarctive foci (local necrosis).

Statistical analysis: DatawereanalyzedbythemeansofSPSSsoftware(statistical packagefor socialsciences)version24,meanwithstandarddeviationwereconsideredas descriptivemeasures whileOne-Way ANOVAwasconsideredtotestsignificant differencesbetweenmorethan2groups,inwhichPostHoc.Tukey testwasusedfor multiple comparisons. Mann-Whitney U test was used to compare histopathological scoresbetween2groups.GraphPadPrismversion8softwarewasusedtodesignthe errorbarchartsfor moreclarificationofdata.Statisticalsignificanceinallthetestswas considered when Pvalue$\leq$ 0.05.

Results

Inorder toevaluate theneuroprotectiveeffectsofcombinationofbothmyricetinand berberine, anumberof inflammatoryand apoptoticparameterswere examined after inductionof global cerebralischemia withandwithoutpretreatmentwiththose agentsin addition to infarction size assessment and histopathological analysis.

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Notch 1 receptor and jagged 1 ligand expressions were also tested to examine the potentialroleofNotchsignalingpathwayinglobalcerebralischemiaandinmediating the proposed drugs neuroprotectiveeffects.

Treatmentcombinationattenuatesinflammatory parametersinduced byCI/ RI: Cerebralischemia/reperfusioninjuryisknowntoinduceaninflammatoryprocess,so thatwewereinterestedtoevaluatetheinflammatory statusofthebraintissueintermofa proinflammatory cytokine;IL-1β,andintercellularadhesionmolecule;ICAM-1andthe proposed protective effect with pre-treatment bymyricetinplus berberine combination.

Effectoncerebralcytokine(IL-1β)level: The cerebralconcentrationofIL-1β was significantly($p<0.05$)elevatedincontrolgroupattheendofthestudy incomparisonto sham group(201.67±2.73 vs. 93.89±0.97 pg/ml), meanwhile control andcontrol-vehicle groupsshowedinsignificantdifferencesbetweenthem.TreatmentcombinationgroupIL-1βcerebralconcentrationwassignificantly ($p<0.05$)lesserthancontrol-vehiclegroup (119.04±2.11 vs.206.84±3.23 pg/ml). Thechanges inIL-1βcerebralconcentration are summarized in figure(1).

EffectoncerebralICAM-1: ThecerebralconcentrationofICAM-1wassignificantly ($p<0.05$) elevated in control group in comparison to sham group (23.67±0.32 vs. 8.89±0.34 ng/ml), while control and control-vehicle groups showed insignificant differencesbetweenthem.TreatmentcombinationgroupICAM-1cerebralconcentration wassignificantly($p<0.05$)lesserthancontrol-vehiclegroup(15.66±0.20vs.22.78±0.26 ng/ml). Thechanges inICAM-1 cerebral concentration aresummarized figure(2). **Treatmentcombinationattenuatescaspase-3inducedbyCI/RI:**Caspase-3 activation is a marker for celldeathand cerebral ischemia/reperfusion injuryis supposed to causean elevationinthecaspase-3levels.Ourresultsconfirmedthatthecerebralconcentrationof caspase-3wassignificantly($p<0.05$)elevatedincontrolgroupincomparisontosham group(6.91±0.21vs.0.82±0.09ng/ml),meanwhilecontrolandcontrol-vehiclegroups showedinsignificantdifferencesbetweenthem.Treatmentcombinationgroupcaspase-3 cerebral concentration was significantly ($p<0.05$) lesser than control-vehicle group (2.91±0.25vs.6.71±0.20ng/ml).Thechangesincaspase-3cerebralconcentrationare summarized in figure(3).

NotchsignalingpathwayisactivatedduringCI/RI: TheNotchpathway isahighly conservedsignaling systemthatcontrolscellularself-renewalandsurvivalduring the developmentofvarioustissues.Therefore,duringcerebralischemia/reperfusioninjury weproposedthat this pathwayis involved in the process brain tissuedamage.

EffectoncerebralNotch1receptor: ThecerebralconcentrationofNotch1receptor wassignificantly($p<0.05$)elevatedincontrolgroupattheend ofthestudyincomparison to sham group(19.14±0.35 vs.14.07±0.37ng/ml), meanwhile control andcontrol-vehicle groups showedinsignificantdifferencesbetweenthem.On the other hand,treatment combinationgroupwassignificantlyhigherthancontrol-vehiclegroup(20.89±0.29vs. 19.28±0.20ng/ml).The changesinNotch1receptorcerebralconcentration are summarized in figure(4).

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EffectoncerebralJagged1ligand: The cerebralconcentrationofJagged1ligandwas significantly($p<0.05$)elevatedincontrolgroupincomparisontoshamgroup(19.05± 0.34 vs. 11.66±0.35 ng/ml), meanwhile control and control-vehicle groups showedinsignificantdifferencesbetweenthem.Ontheotherhand,treatmentcombinationgroup wassignificantlyhigherthancontrol-vehiclegroup(20.41±0.2vs.18.45±0.25ng/ml). The changes in Jagged 1ligand cerebral concentration aresummarizedinfigure (5). **Treatment combination attenuates cerebral infarction size induced by CI/ RI:** Cerebralischemia/reperfusioninjurycausesinfarctionintheaffectedareaofthebrain whichappearsaswhitecolorwhenstained by TTCstainwhilethevalidareaappearedas redcolor.Theinfarctionpercentagewassignificantly($p<0.05$)elevatedincontrolgroup incomparisontoshamgroup(52.98±2.69%compareto0%),whileeachofcontroland control–vehicle groups showed insignificant differences between them. Treatment combinationgroupshowedsignificantdecreaseininfarctionpercentagesincomparison tocontrol-vehiclegroup(17.23±3.07%vs.51.51±2.84%).Thechangesintheinfarction percentagearesummarized infigure(6). **Treatmentcombinationattenuatesbraintissuehistopathologicaldamageinduced byCI/RI:**Damagetothe braintissue appearedunder themicroscopeasnormal,mild, moderate or severetissuedamage dependonthe presentofedema,dark neurons, hemorrhagicarea,or necrosis.Regarding histopathologicalscores,allratsinshamgroup showednormaltissue scores,while controlgroupshowedmoderate damage score in2 samplesandseverein4.Treatmentgroupshowednormalscore in2samples,mildin3 samplesandmoderatein1samplewithnosevere damagescore.Thechangesare summarized in table (1).

Discussion

Differenttherapeuticplanshavebeen suggestedbyexperimentalanimalstudiesand clinicaltrialstoprotectfromthe harmfuleffectsof ischemia reperfusioninjuries. Neuroprotective agentscantargetspecific pathophysiologicalstepin cerebralischemia reperfusioninjury likeoxidativestress,apoptoticcelldeathandsomany others**(23)**.The inhalationofgaseslikehydrogengaswasfoundtobeneuroprotectiveby decreasing oxidative stressand oxygenfreeradicalsformation**(24)**, other inhalationanestheticslike isoflurane and sevoflurane could confer neuroprotection in some animal models of cerebralI/Rinjury**(25,26)**.

Inourstudy,theinflammatory mediatorsIL-1βandICAM-1wereincreasedincontrol group incomparisontoshamgroup.Iadecola andAnratherhave establishedthat inflammation plays a key role in the propagation of ischemic cerebral injury **(27)**. ResidentmicroglialcellsareconsideredthemainsourceofIL-1βinearly braininjury afteranischemicinsult**(28).**Haqqanietal.haveillustratedabiphasicexpressionofIL-1βmRNA,theearly elevationoccurswithin1hourofreperfusion,whilelateelevation occurswithin6-24hoursofreperfusionafter20minutesoftransientglobalcerebralischemia**(29)**.By interactingwithintegrinsexpressedonleukocytessurface,ICAM-1is essentialfor the adhesionof leukocytestoactivatedendotheliumsurface**(30)**.The enhancedexpressionofICAM-1found incerebralendothelial cellsisinvolvedin promoting theinfiltrationofleukocytestothebrainafteranischemicinsultasevidenced byLieszetal.whoshowedsignificantlydecreasedinfiltrationofimmunecellstothe brainafterantiICAM-1antibodiesadministrationinanexperimentalmodelofstroke **(31)**.Wefoundthatdrugstreatment1hourbefore inductionoftransient globalcerebral ischemiasignificantly decreasedproinflammatory cytokineIL-

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1βandintercellular adhesionmolecule(ICAM-1)cerebrallevelsascomparedtocontrol-vehiclegroup,Sun Yetal.studiedtheprotectiveeffectofmyricetininintestinalI/RI inratsinducedby clampingthesuperiormesentericartery for1hourandthenallowingreperfusionfor2 hours,andfoundthat myricetintreatmentintragastrically causedsignificantdose dependentreductioninproinflammatorycytokines**(15)**.Aninvitrostudyconductedby J.-D.Kimetal.on humanumblicalveinendothelialcells(HUVECs)totestthe antioxidant,antiangiogenic andcelladhesioneffectsof flavonoidsdemonstrated that myricetin significantly and dosedependentlydecreased IL-1β induced expression of VCAM-1,ICAM-1,andE-selectin relatingthateffecttothe number of OH- moietieson the β ringin its structure**(32)**.

Caspase-3isthemostabundanteffectorcaspaseinthedevelopingbrainaswellasa potenteffector of apoptosis triggered viaseveraldifferent pathways. Caspase-3 was recognized in animal models ofcerebral ischemiaas amain mediatorofapoptosis. Asashietal.showedthatcaspase-3mRNAwassignificantlyelevatedinratsbrain1hour after pMCAO **(33)**, G. Liu et al**.**also confirmed that cerebral ischemia can trigger neurologicalimpairmentandleadtoneuronalapoptosisthatmaybeconnectedwith caspase-3andBaxactivationandBcl-2downregulation**(34)**.Comparablefindingsin caspase-3 upregulation afterischemiahavebeen expanded to ischemichumanbrain tissue**(35)**.Inourstudydrugstreatmentintraperitonealy1hourbeforeinductionof transientglobalcerebralischemiasignificantlydecreased caspase-3cerebrallevelas comparedtocontrolvehiclegroup.Scarabellietal.demonstratedtheantiapoptoticeffect ofmyricetininmyocardialI/RIinratsinwhichmyricetinreduceddeathreceptorFAS expression and caspase-3 activation by inhibition of STAT1 phosphorylation **(36)**. Comparableresultswerefoundinintestinal I/Rinjuryratmodelinwhichmyricetin reducedcaspase-3and Bcl-2 protein expression whileincreasing BAX expression**(15).** Notch signaling pathwayplays avital rolein preserving NSCs pool and regulating neurogenesisinembryonicandadultbrainbyinhibitingdifferentiationofneuronsto permit consecutive waves of neurogenesis **(37).** Besides its participation in neuraldevelopment,Notchpathway alsoplaysaroleincerebralischemia.XiaoMeiWanget al.demonstratedthatNotch1receptorwasexpressedchiefly inneuronalprecursorcells andimmature neuronswhich isDCX positive,while Jagged1ligandwaspredominantin (GFAP)-positiveastrocyticcellsintheSVZ,they alsoshowedthatNotch1signaling pathway wasactivatedasearly as4hoursofreperfusionaftertMCAOintheSVZ ofrat brainwhichwasassociatedwithenhancedneurogenesisinthe SVZ**(38**).Inour study, bothNotch1receptorand Jagged1ligandwere elevatedincontrolgroupincomparison withshamgroup, butdrugstreatment1hour before inductionof transientglobalcerebral ischemia didn't have significanteffectonbothNotch1andJagged1proteinscerebral levelsascomparedtocontrolvehiclegroup.Sowe propose thatmyricetinandberberine combinationhasneuroprotectiveeffectsarenotrelatedtoNotchpathway regulation. LaFoya,Munroe,andAlbigshowedthatmyricetinneitheractivatednorrepressedNotch

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1signalinginHumanMicrovascularEndothelialCells(HMEC-1)culture**(39).**Tothe bestofourknowledge,thereisnopreviousstudy investigatedtheeffectofmyricetinon Notch signalingpathwayregulation in globalcerebral ischemia.

Measurement ofinfarction size revealedsignificant elevation in infarction size after CI/RI whilecombinationtreatmentcausedsignificantreductioninfarctionsize.

Chandrashekharetal.showedthatglobalcerebral CI/RI significantincreaseininfarction size**(21)**.Inhistopathologicalexamination;we showedsignificantdifferencein histopathological scoresamong control, shamand treatmentgroups.Control group histopathologicalexaminationshowedsevere andmoderatecerebralinjury,shamgroup examination showed normal cerebral histology, while the score of treatment group showednormalbrainhistologyandmildinjury.Thisindicatesthattreatment1hour beforeinductionoftransientglobalcerebralischemiasignificantly amelioratedcerebral injury. Shahetal. demonstrated thatbothfocalandglobal cerebralischemia for 30 minutesinratsproducedcongestionof bloodvesselswhile necrosiswasevidentafter1 hourofreperfusion**(17).**Y.Sunetal.demonstratedthattreatmentinintestinalI/RI modelinratsignificantly reducedthe damage scoreinhistopathologicalexaminationina dosedependentmanner **(15)**.

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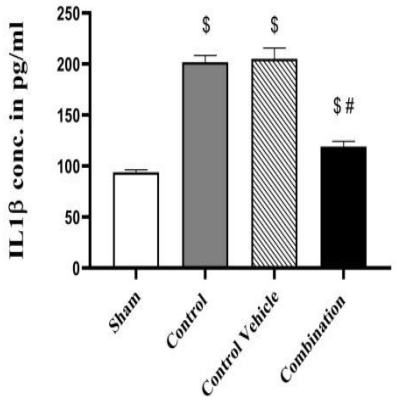
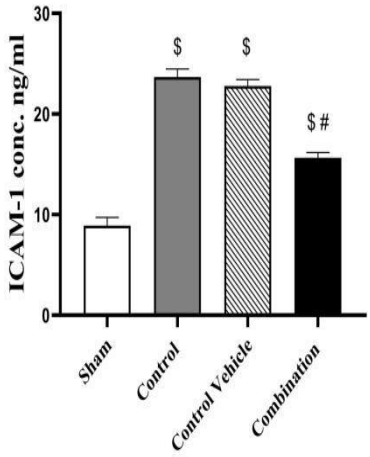


Figure(1): CerebralIL-1βconcentrations(pg/ml) inallexperimentalgroups. Valuesexpressed as mean±SEM ($Vs. Sham, # Vs. Controlvehicle).



Figure(2): CerebralICAM-1 concentration (ng/ml) in allexperimentalgroups. Valuesexpressed as mean ±SEM.($Vs. Sham, # Vs.Controlvehicle).

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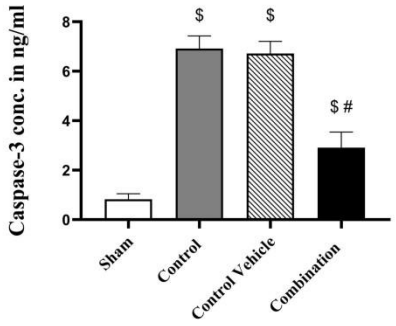
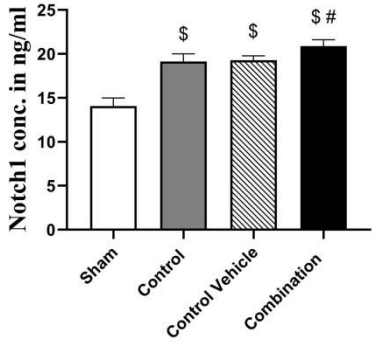
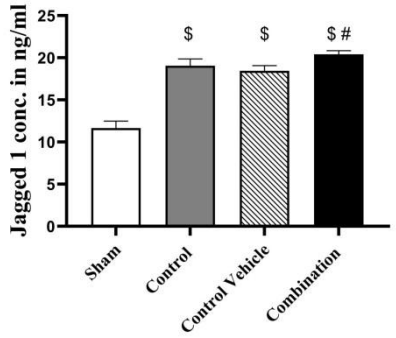
Received 05 March 2021; Accepted 01 April 2021.



Figure(3): Cerebralcaspase-3 concentration(ng/ml)in theexperimentalgroups. Valuesexpressed asmean ±SEM($Vs. Sham, # Vs. Controlvehicle).



Figure(4): CerebralNotch1 concentration(ng/ml) in allexperimentalgroups. Valuesexpressed as mean ±SEM($Vs. Sham, # Vs. Controlvehicle).



Figure(5): CerebralJagged 1 concentration (ng/ml)inallexperimentalgroups. Valuesexpressed as mean ±SEM($Vs. Sham, # Vs. Controlvehicle).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 3163 – 3174
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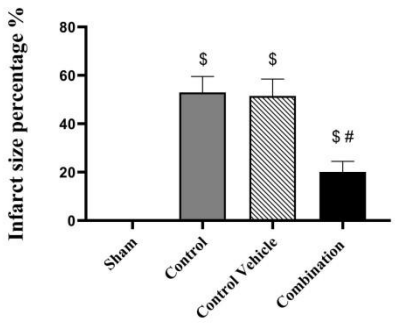

Figure(6): Cerebralinfarction percentages in allexperimentalgroups. Valuesexpressed as mean ±SEM($Vs. Sham, # Vs.Controlvehicle

Histologicalscore	**Groups**							
	Sham		**Control**		**Controlvehicle**		**Combination**	
	n	**%**	***n***	**%**	***n***	**%**	***n***	**%**
Normal	6	100.0	0	0.0	0	0.0	2	33.3
Mild	0	0.0	0	0.0	0	0.0	3	50.0
Moderate	0	0.0	2	33.3	4	66.7	1	16.7
Severe	0	0.0	4	66.7	2	33.3	0	0.0
Total	6	100.0	6	100.0	6	100.0	6	100.0

Table(1): Histopathologicalscoresinallexperimentalgroups.