UNIVERZITET U BANJOJ LUCI

MEDICINSKI FAKULTET

Zoran Matković

EFEKTI LEVOSIMENDANA U AKUTNOJ ISHEMIJI MEZENTERIJUMA KOD PACOVA

DOKTORSKA DISERTACIJA

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FACULTY OF MEDICINE

Zoran Matković

EFFECTS OF LEVOSIMENDAN IN ACUTE ISCHEMIA OF THE MESENTERIUM IN RATS

DOCTORAL DISSERTATION

Banja Luka, 2026.

Mentor: Prof. dr Zoran Aleksić, redovni profesor, Medicinski fakultet Univerziteta u Banjoj Luci

Naslov doktorske disertacije: Efekti levosimendana na akutnu ishemiju mezenterijuma kod pacova

Rezime: Akutna mezenterična ishemija (AMI) je redak uzrok akutng abdomena koji se karakteriše otežanim protokom ili prekidom krvotoka kroz crevo. Princip lečenja je reperfuzija- upostavljanje krvotoka ishemičnog creva ili resekcija nekrotičnih delova creva. Hirurgija i endovaskularne intervencije su dva komplementarna pristupa u tretmanu AMI koji se dopunjuju. Multidisciplinarni pristup je neophodan jer se radi o urgentnom i veoma kompleksnom oboljenju. Različiti lekovi sa antioksidativnim, antiiflamatornim i vazoaktivnim svojstvima se koriste u tretmanu AMI. Levosimendan je lek sa dokazanim brzim antiishemijskim dejstvom koji se koristi u tretmanu akutne srčane insuficijencije. Ova disertacija ima za ciljeve da istraži markere oksidativnog stresa i inflamacije, patohistološke i imunohistohemijske promene u organima tokom AMI, i da pruži moguće strategije lečenja AMI. Ova studija evaluira zaštitne efekte pretretmana levosimendanom na tanko crevo, srce, pluća i bubrege kod pacova u modelu ishemije/ reperfuzije (I/R) gornje mezenterične arterije,. Muški Wistar pacovi (ukupno 24) su podeljeni u 4 jednake grupe: kontrolnu grupu, I/R grupu, levosimendan (LS) 1mg/kg i.p. grupu i grupu IR/LS (1 mg/kg, 30 minuta pre lezije). Rezultati su pokazali da I/R uzrokuje porast oksidativnih markera (reaktivnih supstanci tiobarbiturne kiseline (Thiobarbituric acid reactive substances, TBARS), vodonik peroksida (H_2O_2), superoksid anjon radikala (O_2^-) i azot dioksida (NO_2^-)), indukuje inflamaciju- infiltraciju makrofaga i produkciju interleukina 6 (IL-6) i apoptozu- porast pojačivača lakog lanca kapa nuklearnog faktora aktiviranih B ćelija (Nuclear factor kappa B, NF-kB), cepane kaspaze (cleaved caspase 3, CC3) i TUNEL pozitivnih ćelija (obeležavanje krajeva preloma deoksi nukleotidil transferaze (dUTP) posredovano terminalnom deoksi-nukleotidil transferazom (TdT) (terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labelling, TUNEL)). Pretretman levosimendanom značajno je smanjio markere oksidativnog stresa i pojačao je aktivnost antioksidativnih odbrambenih faktora kao što su katalaza (CAT), redukovani glutation (GSH) i superoksid dismutaza (SOD). Histološke analize su pokazale smanjeno oštećenje sluzokože i očuvanje goblet ćelija u crevu. Slični zaštitni efekti su primećeni i u drugim organima, kao što su srce, pluća i bubrezi. Imunohistohemija je pokazala smanjenu epitelnu apoptozu i bolju regulaciju antioksidativnih i antiiflamatornih proteina kao

što su nuklearni eritroidni faktor 2- povezani faktor 2 (Nuclear factor erythroid 2-related factor 2, Nrf2) i hem oksigenaza-1 (heme oxygenase-1, HO1). Ovi nalazi ističu sposobnost levosimendana da zaštiti tkiva i udaljene organe tokom mezenterične I/R suzbijanjem oksidativnog stresa, inflamacije i apoptoze naglašavajući njegov terapijski potencijal u kliničkim uslovima.

Ključne reči: ishemija/reperfuzija, gornja mezenterična arterija, levosimendan, oksidativni stres, inflamacija, apoptoza

Naučna oblast: Medicinske i zdravstvene nauke

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Klasifikaciona oznaka za užu naučnu oblast prema CERIF šifrarniku: B 600, B 726

Mentor: Zoran Aleksić, MD, PhD, Full professor, Faculty of Medicine, University of Banja Luka

Doctoral thesis: Effects of levosimendan on acute mesenteric ischemia in rats

Summary: Acute mesenteric ischemia (AMI) is a rare cause of "acute abdomen", characterized by a disturbed blood flow in the intestines. AMI still has high morbidity and mortality today. The principle of treatment is the restoration of the perfusion of the ischemic intestine and the resection of necrotic intestine. Surgery and endovascular intervention are two complementary approaches to AMI. Due to the need for urgent and complex treatment of patients with AMI, a multidisciplinary approach is necessary. Different drugs with vasoactive, antioxidant, and anti-inflammatory properties have been used to treat AMI. Levosimendan is a drug with proven anti-ischemic effects used in the management of acute congestive heart failure. This dissertation aims to investigate markers of inflamation and oxidative stress, patohystologic and imunohistochemistry changes on organs during AMI, and highlight the latest studies and provide patient treatment strategies with AMI. This study evaluated the protective effects of levosimendan pretreatment on intestinal, as well as lung, heart, and kidney tissue in a rat model of mesenteric artery ischemia/reperfusion (I/R) injury. Male Wistar rats (N = 24) were divided into four groups: control, I/R, levosimendan (LS) 1 mg/kg i.p, and LS + I/R (1 mg/kg i.p. 30 min before injury). I/R by itself caused elevation of oxidative markers (thyobarbituric acid reactive species (TBARS), hydrogen peroxide (H_2O_2), super oxide anjon radical (O_2^-), and nitrogen dioxide (NO_2^-)), induced inflammation (macrophage infiltration and Interleukin-6 (IL-6) production), and apoptosis (nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), cleaved caspase-3 (CC3), and TUNEL positive cells (terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)). Levosimendan pretreatment significantly reduced oxidative stress markers and enhanced antioxidant defences (catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD)). Histological analysis revealed reduced mucosal damage and preserved goblet cells in intestinal tissue. Similar protective effects of levosimendan were observed in other organs such as lung, heart, and kidney. Immunohistochemistry showed reduced epithelial apoptosis and upregulation of antioxidant and anti-inflammatory proteins, such as Nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1).

These findings highlight levosimendan's ability to protect mesenteric I/R tissue injury and multi-organ damage by suppressing oxidative stress, inflammation, and apoptosis, emphasing its therapeutic potential in clinical settings.

Keywords: ischemia/reperfusion; mesenteric artery; levosimendan; oxidative stress; inflammation; apoptosis

Scientific area: medical et health Sciences

Scientific field: Medical Sciences

Narrow scientific area: Surgery, Pathophysiology

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Sadržaj

1. UVOD

1.1. Defininija, opšta razmatranja

Akutna mezenterična ishemija (AMI) je kritična hirurška, hitna situacija i definiše se kao iznenadna neadekvatnost arterijskog snabdevanja ili venske drenaže creva, što dovodi do ishemije i oštećenja ćelija creva sa ili bez nekroze [1,2]. AMI čini 1 do 2% svih gastrointestinalnih oboljenja. Od 1 000 primljenih pacijenata jedan će imati ovu bolest, ali ima studija koje ukazuju da se taj broj kreće i do 10. Smatra se da 1 od 100 pacijenata sa kliničkom slikom akutnog abdomena zapravo ima AMI [3]. AMI je dakle, kritični i nagli prekid krvotoka ili redukcija protoka krvi u mezenterijumu sa posledičnim funkcionalnim i strukturalnim lezijama u nivou creva i na udaljenim organima [2]. Ovo kompleksno oboljenje najčešće pogađa stariju populaciju sa odmaklom aterosklerozom, sa atrijalnom fibrilacijom, srčanom dekompenzacijom i drugim komorbiditetima. Kod starijih od 80 godina AMI je najčešći uzrok akutnog abdomena, češći nego akutni apendicitis [4,5]. Ova bolest je redak uzrok akutnog abdomena, ali je potencijalno fatalna, vaskularna, hitna situacija sa visokim ukupnim mortalitetom. AMI se češće prepoznaje sa razvojem dijagnostičkih procedura. Stopa preživljavanja se nije značajno poboljšala u proteklih 70 godina, a glavni razlog su stalne poteškoće u prepoznavanju stanja pre razvoja ireverzibilnih promena zida creva, odnosno infarkta creva [6].

Klinička slika je u većini slučajeva nespecifična i može se odlikovati početnim neskladom između jakog bola u trbuhu i minimalnih kliničkih nalaza. Fizikalni pregled ne pravi pouzdanu razliku između ishemijskog i infarktnog creva. Komplikacije kao što su ileus, peritonitis, pankreatitis i gastrointestinalno krvarenje mogu prikriti početne znakove i simptome AMI. Faktori rizika za AMI i klinički tok razlikuju se u zavisnosti od osnovnog patološkog stanja [6-9]. Kako ishemija creva brzo napreduje do ireverzibilne nekroze creva, u roku od nekoliko sati, nastaju teški metabolički poremećaji, što dovodi do niza događaja koji kulminiraju razvojem sindroma multiorganske disfunkcije (*Multiple Organ Dysfunction Syndrome,* MODS) i smrću. Pravovremena upotreba dijagnostičkih i terapijskih metoda za brzo obnavljanje protoka krvi ključ je za smanjenje visoke stope smrtnosti povezane s AMI [2,6,7-9].

Brojni patofiziološki mehanizmi mogu dovesti do mezenterične ishemije, najčešći su: aritmije, (atrijalna fibrilacija), odmakla ateroskleroza, stanja hiperkoagulabilnosti, neoplastični procesi i karcinomatoza, nodozni panarteritis, sistemski lupus, hemolitičko-uremijski sindrom... Okluzivna mezenterična ishemija je posledica arterijske ili venske tromboze ili embolije. Neokluzivna nastaje zbog akutne insuficijencije cirkulacije, obično kod kritično bolesnih pacijenata u jedinicama intenzivnog lečenja (*Intensive Care Unit*, ICU) [10-12]. Neokluzivna mezenterična ishemija (NOMI) se može javiti u stanjima kritične bolesti-šoka, i tada je NOMI posledica upotrebe vazoaktivnih lekova zbog splanhničke vazokonstrikcije usled centralizacije krvotoka dejstvom beta adrenergičkih lekova [13]. Svaki od ovih procesa (okluzivnih i neokluzivnih) uzrokuje sindrom sistemskog inflamatornog odgovora (*Systemic Inflammatory Response Syndrome,* SIRS) ili mezenteričnu ishemijsku nekrozu, što dovodi do ozbiljnog metaboličkog poremećaja i MODS-a [14].

1.2. Incidenca AMI

Za AMI se kaže da je retko stanje, ali je incidenca slabo dokumentovana. Nekoliko studija se njime bavilo među opštom populacijom ili pacijentima koji se nalaze u bolnici, pa su se nedavne smernice, stoga, oslanjale na procenjene nivoe [15]. Nije dostupna sistematska analiza incidence AMI. Najtačniji izveštaj o proporciji različitih oblika AMI dolazi iz populacije sa 87% stope autopsije proučavane između 1970. i 1982 godine. Incidenca u ovim istraživanjima iznosi 6.2/100 000 [16]. Novije studije pokazuju nešto veću incidencu. Najveći broj slučajeva je bio u studiji Crawford-a iz 2016 godine, ukupno 2255, a obuhvatila je period od 2008 do 2013 godine u Merilendu (SAD) gde je učestalost iznosila 10/100 000. Kad se posmatrao broj hospitalizovanih pacijenata učestalost je iznosila 1/1000 primljenih [17].
Kvantitativna sinteza incidence različitih oblika AMI u studiji iz Finske u periodu 2009-2013 godine, pokazala je da incidenca iznosi 8,6 slučajeva okluzivnog arterijskog AMI, 2 slučaja NOMI i 1,8 i 0,5 slučajeva mezenterijalne venske tromboze (MVT) na 100 000 stanovnika. Autori su našli da je okluzivni oblik činio 2/3 svih slučajeva [16,18]. Porast incidence može da se pripiše dužem životnom veku stanovništva. Studije pokazuju da je najčešći oblik okluzivne arterijske AMI, čineći 68,6% svih slučajeva AMI. NOMI čini 15,1% i MVT 11,5% slučajeva. Embolijska AMI (EAMI) je tip koji se izaziva u eksperimentalnim studijama klemovanjem SMA.

Pojedinačne studije pokazuju da se pojava AMI povećava sa godinama. U švedskoj populaciji okluzivni arterijski AMI dramatično se povećava sa godinama i kod muškaraca i kod žena,

dostižući 85,8 na 100 000 osoba u dobu od 80 do 84 godine i 189,5 kod osoba starijih od 85 godina, respektivno [19].

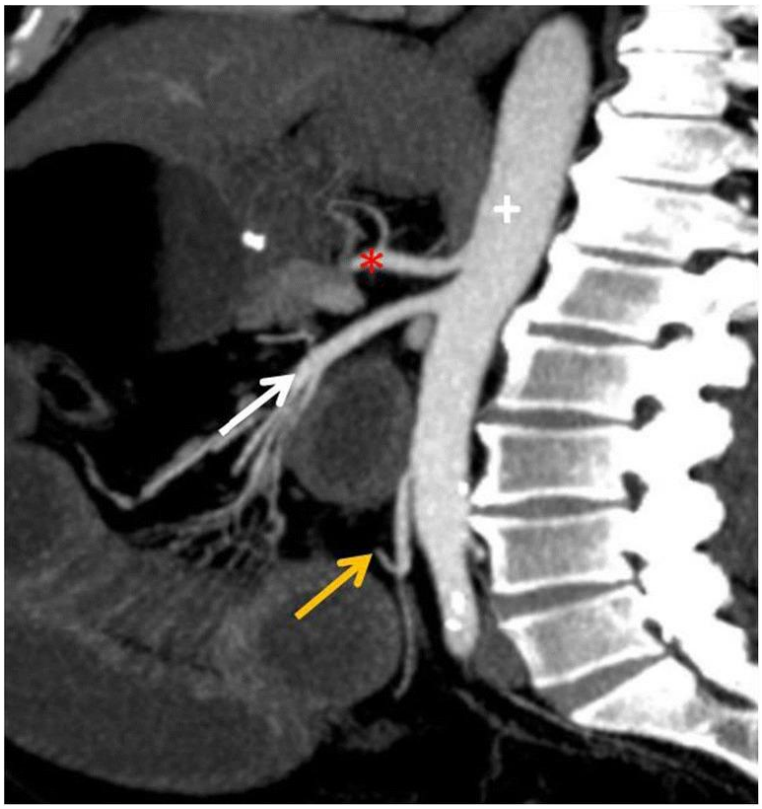
1.3. Mortalitet AMI

Dok su se ishodi drugih vaskularnih katastrofa, kao što su akutni koronarni sindrom i moždani udar, značajno poboljšali tokom proteklih nekoliko decenija, mortalitet nakon AMI ostaje visok, iako je uočeno izvesno blago poboljšanje. Za razliku od incidence podaci o mortalitetu su bolje dokumentovani. Schoots i saradnici su izračunali objedinjeni bolnički mortalitet od 73% iz 47 studija objavljenih od 1967. do 2002 [20]. Adaba i saradnici su došli do rezultata od 63% analizom 52 članka objavljena od 1956. do 2012. Mortalitet je i veći jer se retko rade obdukcije umrlih u starijem životnom dobu u većem delu sveta. Sistematska analiza 45 studija objavljenih pre 2002. godine pokazuje ukupnu smrtnost u bolnicama (hospitalni mortalitet) od 64% ili 74% u zavisnosti od toga da li je primenjen hirurški tretman ili samo potporna terapija [21].

Monita u svojoj studiji iz 2023 godine navodi da se mortalitet i dalje kreće između 60 i 80% [22]. Neki autori navode nešto manji mortalitet, oko 55%, ali se radilo o manjim uzorcima i istraživanje je obuhvatilo hospitalizovane pacijente [23]. Male razlike u mortalitetu u opštoj populaciji i među hospitalizovanima govore da se i dalje kasni sa dijagnostikom i revaskularizacijom što su istakli Tran i saradnici u svojoj studiji [24]. Podaci iz protekle decenije pokazuju da je do određenog poboljšanja moglo doći kao rezultat multidisciplinarnog pristupa i razvoja u mnogim medicinskim oblastima (npr. bolja dijagnostika, endovaskularne procedure, lečenje sindroma kratkog creva, kućna parenteralna ishrana), kao i razvoju centara za intestinalni udar (*Intestinal Stroke Centre*, ISC) [25-27].

1.4. Anatomija mezenterijalne vaskularizacije

Tri glavne arterije koje snabdevaju tanko i debelo crevo su celijačno stablo (*truncus coeliacus*, TC), gornja mezenterična arterija (*Superior Mesenteric Artery*, SMA) i donja mezenterična arterija (*Inferior Mesenteric Artery*, IMA) (Slika 1). TC osigurava vaskularizaciju od distalnog jednjaka do drugog dela duodenuma. SMA snabdeva treći i četvrti deo duodenuma, jejunum, ileum i ushodni i poprečni deo debelog creva do nivoa lijenalne fleksure. IMA snabdeva distalni kolon od nivoa lijenalne fleksure do gornje trećine rektuma.



Slika 1. Anatomija: Sagitalna CT MIP (projekcija maksimalnog intenziteta) slika prikazuje tri glavne arterije koje opskrbljuju creva, celijačno stablo (TC) (zvezdica), gornju mezenteričnu arteriju (SMA) (bela strelica) i donju mezenteričnu arteriju (IMA) (narandžasta strelica), koje su visceralne grane abdominalne aorte (+).

Postoje brojni važni mezenterični kolateralni putevi koji pružaju bogatu vaskularnu sigurnosnu mrežu za opskrbu mezenterijskom krvlju. Gastroduodenalna arterija, obično prva grana zajedničke hepatične arterije, predstavlja važan kolateralni put između celijačnog stabla (TC) i SMA. Drummondova marginalna arterija (marginalna arterija debelog creva) i Riolanova arkada (arkada crevne arterije) povezuju SMA i IMA. Četiri arkade anastomoze formiraju se između IMA i lumbalnih arterija koje potiču iz aorte, sakralne i unutrašnje ilijačne arterije. Osim toga, periferni mali krvni sudovi su anatomski raspoređeni u paralelnu serijsku konfiguraciju koja snabdeva mukozu, submukozu i duboki mišićni sloj (*lamina muscularis propria*) creva [28].

1.5. Fiziologija mezenterijalne cirkulacije

U normalnim okolnostima, ljudsko crevo prima oko 20%-25% minutnog volumena u mirovanju, od čega dve trećine snabdeva crevnu sluznicu. U postprandijalnoj fazi, splanhnička autoregulacija može povećati protok krvi u crevima do čak 35% minutnog volumena srca [29,30].

Autoregulacija intestinalne perfuzije može održati vitalnost tkiva ispod sistemskog krvnog pritiska od 70 mmHg. U slučajevima kada je sistemski krvni pritisak ispod 40 mmHg, lokalna miogena autoregulacija je nadjačana sistemskom autoregulacijom i lokalni zaštitni mehanizmi ne uspevaju da održe vitalnost. To dovodi do sve veće ishemije crevnog zida, slično onome što bi se dogodilo u slučaju mezenterične hipoperfuzije zbog vaskularne okluzije. Početno ishemijsko oštećenje crevnog zida tada može da varira od samo blage i površinske nekroze ograničene na sluznicu (stadijum I), ili oštećenja koja se protežu na slojeve submukoze i mišićnog sloja (stadijum II), do opasne i po život opasne kontinuirane nekroze svih slojeva crevnog zida (transmuralni infarkt creva) (stadijum III) [31,32].

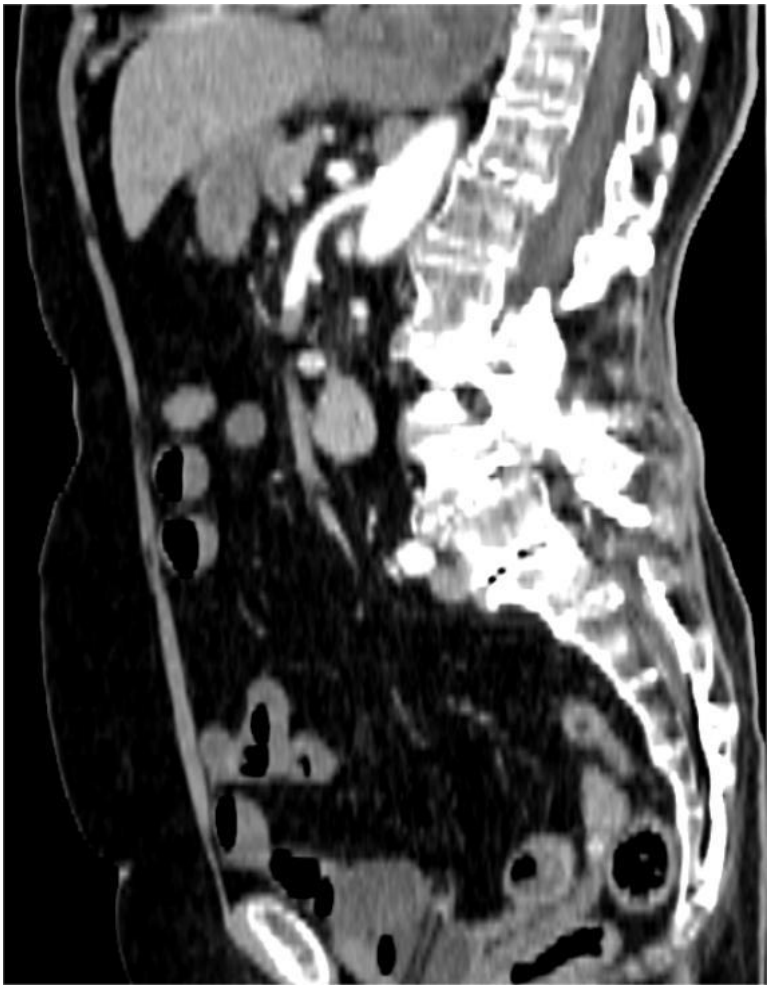
1.6. Oblici AMI

AMI se može podeliti na okluzivni oblik i neokluzivni oblik (NOMI).

Okluzivni oblik obuhvata:

- Embolizaciju arterije mezenterike superior (EAMI) u 40-50% slučajeva,
- Trombozu SMA u 25-30% (TAMI), Akutni arterijski trombi i embolusi mogu se pojaviti kao očigledni nedostaci punjenja u lumenu krvnog suda.
- Mezenterijalnu vensku trombozu (MVT) u 10-18% (kod maligniteta, nekrotizirajućeg pankreatitisa, mijeloproliferativne bolesti, deficit proteina C i S).

Neokluzivni oblik (NOMI) čini do 20% svih slučajeva AMI i sreće se kod kritično obolelih [33]. Razvoj intestinalne ishemije usled arterijske opstruktivne lezije zavisi od lokacije opstrukcije, razvoja kolateralnih krvnih sudova i stepena opstrukcije (slika 2). Kao što je već rečeno, prisustvo kolateralnih arkada omogućava dvosmerni protok, koji može da zaobiđe opstruktivne lezije. U prisustvu opstrukcija koje zahvataju sve tri glavne arterije (TC, SMA i IMA), frenične, lumbalne i karlične kolateralne arterije mogu se proširiti kako bi osigurale pomoćni visceralni protok krvi. Kada je lezija distalno od tačke kolateralnog toka, kolateralni dotok krvi nije moguć i najverovatnije će doći do ishemije.



Slika 2. Okluzija SMA embolusom (EAMI) na nekoliko cm od aorte, distalno od račvanja donje pankreatikoduodenalne arterije. Sagitalna projekcija, CT prikaz.

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1.7. Patofiziologija AMI

Proces razvoja AMI je izuzetno složen. Ukratko, sa patofiziološkog gledišta, početne ishemijske lezije creva obično su praćene oslobađanjem određenih medijatora kao što su citokini, adhezioni molekuli, faktor aktivacije trombocita i faktor nekroze tumora, što će dovesti do upalnog odgovora i oštetiti zid creva. Dodatno oštećenje creva nastaje sa oslobađanjem reaktivnih kiseoničnih vrsta, (*Reactive Oxygen Species*, ROS) i reaktivnih azotnih vrsta, (*Reactive Nitrogen Species*, RNS) [34,35]. Kao posledica prekida krvotoka tokom nekoliko sati, dolazi do transmuralne nekroze, mukozna barijera se oštećuje i crevo gubi otpornost na invaziju bakterija, što dovodi do bakteriemije i sepse [36].

Tri najvažnije komponente patofiziološkog procesa su oksidativni stres, inflamacija i apoptoza. Kaskada procesa pojednostavljeno se kreće ovako:

-Hiperprodukcija ROS

-Oštećenje biomolekula

-Mitohondrijalna disfunkcija

-Regrutovanje imunskih ćelija

-Oštećenje antioksidativnih odbrambenih sistema

-Aktivacija inflamatornih i apoptotskih puteva.

1.7.1. Oksidativni stres u AMI i I/R leziji creva

Ishemijsko-reperfuzijska lezija (I/R) je manifestacija oštećenja tkiva ili organa koje je praćeno ishemijom i pogoršano povratkom krvotoka u prethodno oštećeno tkivo ili organ. Creva su jedno od najosetljivijih tkiva i organa na I/R lezije. Osim toga, štetne posledice I/R lezije creva nisu ograničene na samo crevo i mogu dovesti do oštećenja udaljenih tkiva i organa (MODS). Mehanizam I/R je veoma kompleksan niz procesa koji se mogu međusobno i ubrzavati i kočiti preko signalnih puteva, a u kojima je oksidativni stres ključna karika u patogenezi I/R lezije [37].

U eksperimentalnim studijama i kliničkoj praksi, sama ishemija ne nanosi veliku štetu organizmu; umesto toga, višak ROS uništava ćelije i čvrste spojeve (*tigh junction*) između ćelija, a kako se obnavlja protok krvi i ovaj šok direktno uzrokuje dalje oštećenje čitavog organizma [38]. Intestinalna I/R lezija je vrlo ozbiljna patofiziološka pojava. Ova lezija je često uzrokovana i hirurškom problematikom, teškom traumom, opekotinama, šokom, intestinalnom torzijom, inkarceracijom, operacijama abdominalne aorte, disekcijom aorte i SMA, transplantacijom tankog creva i hemoragično-nekrotičnim pankreatitisom [37-39].

Intestinalna I/R je patološki događaj opasan po život, koji nije ograničen na creva i može molekularnim mehanizmima kao što je metilacija DNK u jedrima ćelija i u mitohondrijama da uzrokuje sistemske poremećaje SIRS i MODS, koji su usko povezani s visokom incidencom i stopom smrtnosti AMI [40]. Mehanizmi uključeni u intestinalnu I/R su prilično složeni i odgovor crevne sluznice na I/R može se podeliti u sledeće dve faze: prvo, hipoksija tkiva i oštećenje organa nastaju tokom ishemije; drugo, velike količine ROS-a nastalih tokom reperfuzije krvotoka i reoksigenacije pokreću reakciju oksidativnog stresa, što potom dovodi do razaranja crevne mukozne barijere, povećane vaskularne permeabilnosti, translokacije bakterija i oslobađanja inflamatornih medijatora i apoptotskih faktora [41].

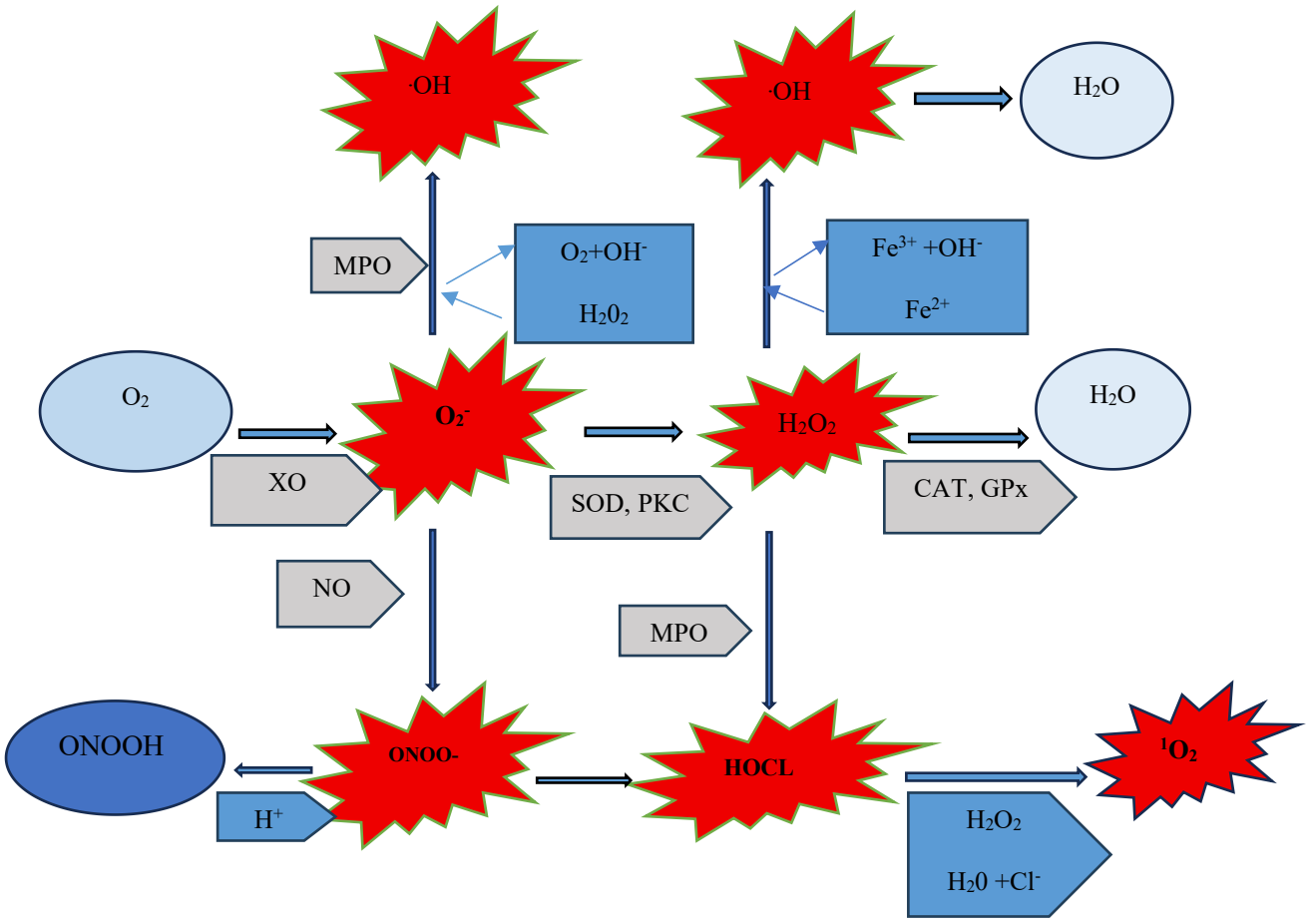
Poslednjih godina, sve više dokaza ukazuje da oksidativni stres igra ključnu ulogu u patogenezi I/R. Kako se dotok kiseonik obnavlja tokom reperfuzije, obilni ROS u oštećenim ćelijama i tkivima može napasti gotovo sve intracelularne biomolekule, npr. ćelijske membrane, organele (mitohondrije, endoplazmatski retikulum), uključujući i DNK. Ovaj oksidativni stres remeti dinamičku homeostazu epitelnih ćelija putem prenosa signala, što rezultira oslobađanjem velikih količina inflamatornih medijatora, indukcijom apoptoze i pogoršanjem oštećenja nakon reperfuzije [42]. Mitohondrijska DNK (mtDNA) je uključena u oksidativnu fosforilaciju ćelija i održava normalnu mitohondrijalnu funkciju. Nakon što je mtDNA poremećena, produkcija ROS se povećava i oslobađanje mtDNA u citoplazmu indukuje aktivaciju proinflamatornih medijatora i proapoptotskih faktora [43]. Kako su mehanizmi intestinalne I/R lezije detaljnije istraženi, antioksidativni putevi i zaštita mitohondrija od oštećenja su se pojavili kao važna sredstva za prevenciju i lečenje I/R lezije tokom AMI.

Molekuli kiseonika (O_2) su neophodni za vitalnost i održavanje kondicije organizma i obezbeđuju ATP telu kroz respiratorni kompleks (lanac) mitohondrija čime se obezbeđuje aerobni metabolizam kroz oksidativnu fosforilaciju [44]. Normalni ćelijski metabolizam proizvodi ROS, koji su u malim ili umerenim količinama korisni za neke fiziološke procese, kao što su ekspresija gena, prenos signala, brzina rasta ćelija, razvoj i diferencijacija ćelija, imunske funkcije, adaptivni odgovor- efikasna odbrana od mikroorganizama (oksidativni eustres). Prekomerna proizvodnja ROS-a tokom I/R dovodi do oksidacije tkiva i oštećenja epitelnih ćelija creva. ROS potiču uglavnom iz gastrointestinalnog trakta i iako su crevne epitelne ćelije zaštićene mukoznom barijerom, patogeni mogu proizvesti faktore inflamacije aktivacijom epitelnih ćelija, polimorfonuklearnih neutrofila i makrofaga, a ove inflamatorne ćelije oslobađaju ROS i slobodne radikale u odbrani od patogena. ROS i slobodni radikali se

proizvode kako bi uništili invazivne patogene, ali proizvodnja enormnih količina ROS negativno utiče na homeostazu organizma i dovodi do oksidativnog oštećenja tkiva, autodestrukcije kroz poremećaj jonskih pumpi i influks jona kalcijuma kao i dejstvom fosfolipaze A2 i lipidnom peroksidacijom [45-47].

ROS uključuju jedinjenja slobodnih radikala i neradikalna jedinjenja kao što su: superoksid anjon radikal (O_2^-), hidroksil radikal ($\cdot OH$), vodonik peroksid (H_2O_2), hidroksilna grupa(OH^-). Azotna jedinjenja (RNS) kao što su: azot monoksid (NO), azot dioksid (NO_2), azot trioksid ($N_2O)_3$ i peroksinitrit ($ONOO^-$) su obično blisko povezane sa ROS. Ovi radikali sadrže nesparene elektrone i stoga su visoko hemijski reaktivni prema intracelularnim proteinima, lipidima i DNK. Aktivacija ROS može nepovratno oštetiti i deaktivirati različite makromolekule. ROS mogu biti proizvedene u mitohondrijama, endoplazmatskom retikulumu, ćelijskoj membrani, citoplazmi, jedru i peroksizomima, a mogu se čak proizvesti i kroz ekstracelularni stres [48,49]. Većinu ROS proizvode mitohondrije sisara. U normalnim uslovima, molekule kiseonika redukuje mitohondrijski citohrom c, čime se formira voda i samo nekoliko molekula kiseonika, formiraju se ROS u manjim količinama, na fiziološkom, signalnom nivou pri čemu utiču na gore spomenute funkcije. U normalnim uslovima u telu, ovi slobodni radikali se neutrališu endogenim antioksidativnim enzimima i stoga nemaju štetne efekte na organizam [50].

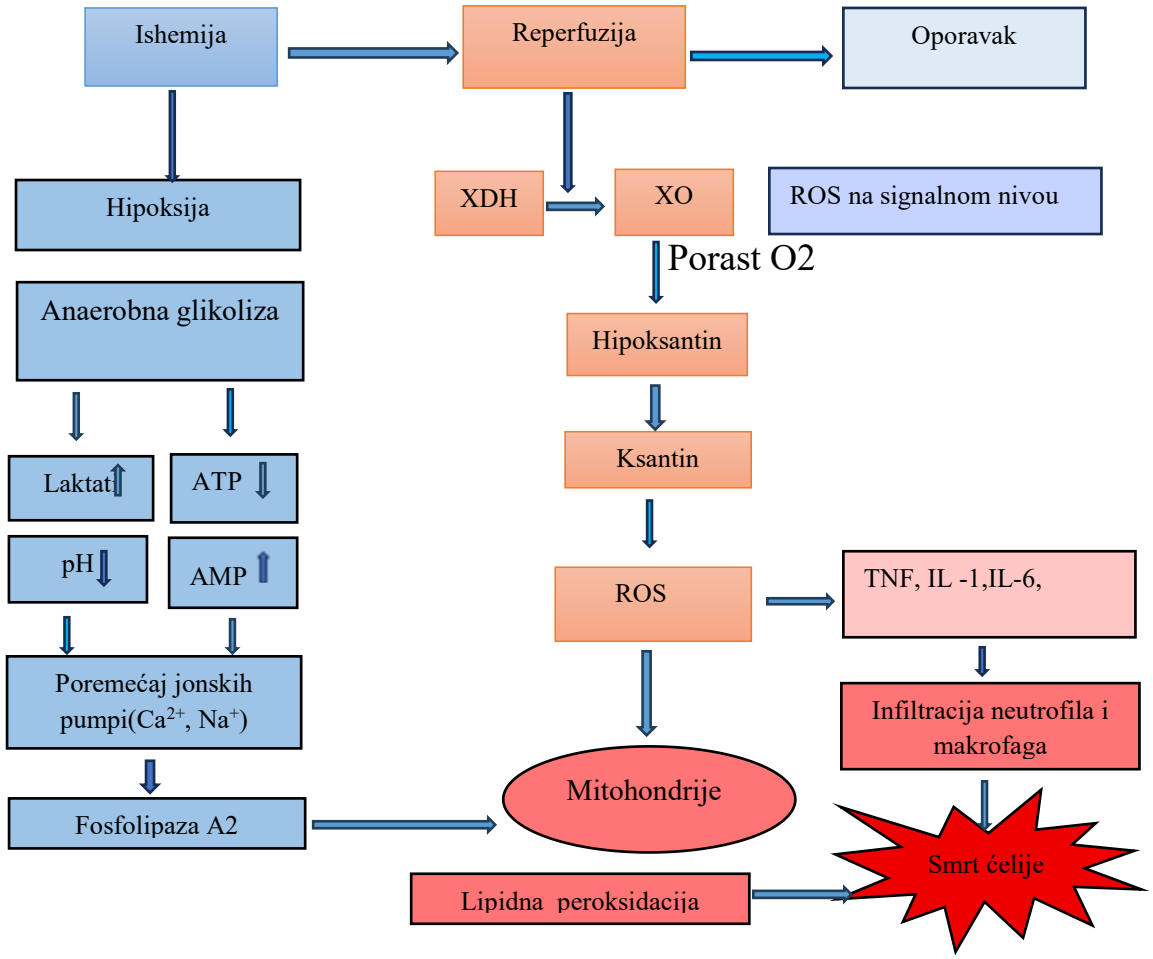
Mitohondrijski respiratorni lanac reguliše proizvodnju ROS. Mitohondrijski kompleksi I i III stvaraju ROS kroz curenje elektrona tokom oksidacije. Mitohondrijska NADPH oksidaza i ksantin oksidaza(*Xanthine Oxydase*, XO) katalizuju konverziju O_2 u O_2^-, mijeloperoksidaza (MPO) vrši konverziju O_2 u OH^-, dok superoksid dismutaza (*Superoxide Dysmutase, SOD)* konvertuje O_2^- u H_2O_2, a mitohondrijalna protein kinaza C *(Protein Kinase C*, PKC) katalizuje konverziju O_2 u H_2O_2. Katalaza (*Catalase*, CAT) potom dalje razgrađuje H_2O_2, slika 3. Enzimi koji kataliziraju hemijske reakcije koja stvaraju ROS *in vivo* takođe uključuju lipoksigenaze, glukoza oksidaze, sintaze azotnog oksida (*Nitric Oxide Synthase*, NOS) i ciklooksigenaze (*Cyclooxygenase,* COX) [51-53].



Slika 3. Formiranje ROS i mehanizmi detoksikacije. SOD: Superoksid dismutaza, CAT: Katalaza, PKC: Protein kinaza C, MPO: mijeloperoksidaza, GPx: Glutation peroksidaza, XO: Ksantin oksidaza, O2: Kiseonik, H_2O_2. Vodonik peroksid, NO: Azot oksid, ONOOH: Peroksinitrozna kiselina, $ONOO^-$: Peroksinitrit, HOCL : Hipohlorit, $^1O_2$: Singletni kiseonik, Cl^- anjon hlora, O_2^-: Superoksid anjon radikal, OH^-: Hidroksilna grupa, ·OH: hidroksil radikal., Fe^{2+}: gvožđe(II)jon, Fe^{3+}: Gvožđe(III) jon, H^+: Jon vodonika, H_2O: voda.

Intestinalna ishemija može dovesti do hipoksičnog stanja koje uključuje promenu u ireverzibilnoj konverziji ksantin dehidrogenaze (*Xanthine Dehydrogenase*, XD) u XO, tokom kojeg se formira reaktivni kiseonik [54]. Nakon što reperfuzija počne i dotok kiseonika se obnovi, elektroni iz XO se prenose na molekularni kiseonik, stvarajući tako značajne količine radikala bez kiseonika kao što su: O_2^-, ·OH i vodonik peroksid (H_2O_2), koji su kratkog veka i visoko reaktivni te brzo oštećuju DNK, ćelijske membrane i organele [55]. Stoga, povreda u intestinalnoj I/R može smanjiti visinu resica, povećati ćelijsku infiltraciju i pogoršati ljuštenje sluznice što se lako uočava histološkim pregledom. Osim toga, proinflamatorni citokini

uključujući faktor nekroze tumora-α (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β) se oslobađaju tokom AMI i izazivaju sistemsku inflamaciju, slika 4 [56].



Slika 4. Patofiziološki procesi tokom I/R lezije. XDH: Ksantin dehidrogenaza, XO: Ksantin oksidaza, ROS: Reaktivne kiseonične vrste, ATP: Adenozin trifosfat, AMP: Adenozin monofosfat, TNF: Faktor tumorske nekroze, IL-1: Interleukin 1, IL-6: interleukin 6. Ca^{2+}: joni kalcijuma, Na^{+}: joni natrijuma.

Iako se veće količine ROS-a proizvode tokom oksidativnog stresa, antioksidansi tela mogu donekle zaštititi ćelije i tkiva od njihovog napada. Enzimski antioksidansi kao što su: SOD, glutation peroksidaza (*Glutathione Peroxidase*, GPx), glutation reduktaza (*Glutathione Reductase*, GSR), CAT i peroksid reduktaza (*Peroxyde Reductase*, PRx) i hem oksigenaza (*Heme Oxygenase*, HO), kao i neenzimski antioksidansi, nekatalitički, antioksidativni proteini glutation (*Glutathione,* GSH), tioredoksin (*Thioredoxin,* TRX) i melationin igraju važnu ulogu u oksidativnom stresu i održavanju homeostaze. U endogene antioksidanse spadaju i mokraćna kiselina, lipoinska kiselina i bilirubin [57].

Egzogene supstance, kao što su vitamin C (askorbinska kiselina), vitamin E (tokoferol), karotenoidi, prirodni flavonoidi, mineralni joni (Mn i Cu) i polifenoli, takođe deluju kao antioksidansi. Vitamin C, koji je hidrofilan, spada u najefikasnije antioksidanse, dok je vitamin E takođe efikasan, a lipofilnost mu omogućava dejstvo inhibicijom lipidne peroksidacije. Praktično sve supstance koje imaju tiolne grupe mogu da neutrališu ROS putem redukcije. SOD i CAT igraju glavnu ulogu u antioksidativnom odbrambenom sistemu. Antioksidansi uklanjaju prekomerne ROS i slobodne radikale tokom reakcija peroksidacije u telu a korišćeni su često u eksperimentalnim studijama i mogli bi da se koriste za lečenje I/R oštećenja [58,59].

1.7.1.1. Signalni Putevi koji pogoršavaju oksidativni stres

Nuklearni faktor-kapa Beta (NF-kB) put signalizacije je posredovan *Toll-like* receptorima (TLR). Radi se o faktoru koji vezuje promotor lakog lanca imunoglobulina kB u beta limfocitima. *Toll-like* receptori su porodica receptora eksprimiranih u ćelijskim membranama i povezanih sa prepoznavanjem patogenih mikroorganizama od strane imunskog sistema, omogućavajući na taj način prenos ekstracelularnih informacija o prepoznavanju antigena u ćeliju [60,61]. Kao receptori za prepoznavanje uzoraka, TLR prepoznaju molekularne obrasce povezane s patogenom (*Pathogen Associates Molecular Patterns*- $PAMP_s$). PAMPs su molekulske strukture mikroorganizama, koje organizam prepoznaje kao strane, putem receptora urođenog imunskog sistema. To su egzogeni ligandi za TLR, kao što su virusi, bakterije, gljivice i drugi patogeni mikroorganizmi. Organizam prepoznaje i molekularne obrasce povezane s oštećenjem (*Damage-Associated Molecular Patterns*, DAMPs). $DAMP_s$ su štetni molekuli oslobođeni iz oštećenih ćelija koji aktiviraju urođeni imunski sistem u interakciji sa receptorima za prepoznavanje obrazaca (*Pattern Recognition Receptors*, PRRs). To su endogeni ligandi za TLR, koje organizam aktivno luči ili pasivno oslobađa tokom stresa. Molekularni obrasci mogu da budu lipoproteini niske gustine i proteini šoka, apoptotske ćelije i fragmenti nukleinske kiseline. Tokom intestinalne I/R prekomerni nivo ROS stimulira aktivaciju TLR-a i posledično pogoršava inflamatorni odgovor [62,63]. Aktivirani TLR pokreću sistemski inflamatorni odgovor vezivanjem na C-terminalni kraj spojnog proteina MyD88 (*Myeloid Differentiation primary response gene 88,* MyD88), a zatim na nishodne ciljne gene, mitogen-aktiviranu protein kinazu *(Mitogen activated protein kinase*, MAPK) i NF-κB preko N-terminalnog kraja molekula MyD88 čime se izaziva oslobađanje inflamatornih medijatora [64]. Deksmedetomidin može da smanji I/R leziju creva upravo inhibibicijom ovog signalnog puta (TLR4/MyD88/NF-κB) kod pacova [65].

U većini modela intestinalne upale, TLR4 indukuje upalne odgovore češće nego drugi TLR. U uslovima izraženog stresa, NF-κB signalni put predstavlja centralni regulator inflamatornog odgovora: oksidativni stres i citokini aktiviraju IκB kinazni kompleks (IKK) što dovodi do degradacije inhibitora kinaze B (IκBα) i translokacije NF-κB p65/p50 heterodimera u jedro. Podjedinica p65 je zadužena za pokretanje transkripcije, dok je podjedinica p50 zadužena za poboljšanje vezivanja NF-kB za DNK. NF-kB se inače sastoji od pet proteinskih podjedinica: p65 (ReL A), p105/p50, p100/p52, ReL B i c-Rel. U jedru NF-kB stimuliše transkripciju proinflamatornih gena poput TNF-α, IL-1β i IL-6, adhezionih molekula, kao i proapoptotskih

faktora Bax (*Bcl-2 Associated X-protein*) i kaspaze-3 [66,67]. Tada dolazi i do aktivacije inducibilne nitrat oksidaze (iNOS) i ciklooksigenaze 2 (COX-2) što pojačava inflamatorni odgovor putem većih količina NO2 i stvaranja peroksinitrita i putem oslobađanja prostaglandina. Aktivacija NF-κB u intestinalnom tkivu tokom I/R lezije u skladu je sa povećanim stepenom apoptoze i smanjenjem ekspresije antiapoptotskog proteina Bcl-2. Fosforilacija IkB koja se dešava pod uticajem IKK je važan korak u aktivaciji NF-kB. Aktivacija IKK je strogo regulisana i specifična za određeni stimulus i predstavlja osnovu za mnoge funkcije povezane sa NF-kB [68]. S druge strane, inhibicija IKK je takođe jedan od glavnih regulatora ovog signalnog puta. Inhibicija inhibitornog procesa IkB kinaze, (supresija IKK kompleksa), može da bude značajan terapijski pristup u prevenciji i lečenju MODS-a razvijenog tokom oksidativnog stresa i inflamacije [69,70]. Dokazano je da pretretman cinamaldehidom smanjuje oksidativni stres obnavljanjem nivoa SOD, GSH, laktat dehidrogenaze (LDH) i malondialdehida (MDA) u crevnim tkivima nakon I/R lezije, takođe ublažava intestinalnu leziju inhibiranjem ekspresije p65 i p50 u NF-κB putu [71]. Inflamatorni odgovori i apoptoza imaju veoma štetne efekte na ćelije i tkiva u I/R leziji. Inhibicija TLR puteva može biti dobar izbor za lečenje I/R lezije.

Tokom oksidativnog stresa, kontinuirano povećanje oštećene mtDNK uništava funkciju mitohondrija. Nivoi kopije mtDNA i transkripcije gena se smanjuju, što utiče na formiranje kompleksa respiratornog lanca i dovodi do mitohondrijalne disfunkcije. Mitohondrijska disfunkcija, zauzvrat, podstiče masivnu proizvodnju ROS, u ciklusu koji na kraju dovodi do apoptoze. Tokom I/R lezije, telo pokreće molekularne obrasce povezane s oštećenjem, od kojih su mnogi izvedeni iz mitohondrija [72-74]. Normalno, mtDNA je prisutna između unutrašnje i spoljašnje mitohondrijske membrane. Tokom intestinalne I/R, mitohondrije su stimulisane oksidativnim stresom i fragmenti mtDNA se oslobađaju u citoplazmu, čime se pokreće niz inflamatornih odgovora [75]. Nekoliko studija je pokazalo da oštećenje mtDNA rezultira oslobađanjem inflamatornih faktora (IL-1β, IL-6 i TNF-α) preko TLR-a i signalnog puta NOD-like receptor proteina 3 (*Nucleotide-binding oligomerization domain-Like Receptor Pyrin domain containing 3*, NLRP3) [76]. NF-kB može da pokrene inflamaciju i uticajem na ovaj inflamazom koji se aktivira odgovorom na DAMP i PAMP pri čemu su uključene kaspaze. NLRP3 inflamazom je citosolni proteinski kompleks u kojem interakcija sa adapterskim proteinom (*Apoptosis -associated Speck-like protein containing a Caspase recruitment domain*, ASC) dovodi do prelaska prokaspaze 1 u kaspazu 1. Ova kaspaza cepanjem prekursora proinflamatornih interleukina (pro interleukini, pro IL 1 i pro IL 18)

dovodi ove interleukine u zrelu i biološki produktivnu formu koji svojim delovanjem izazivaju ćelijsku smrt u urođenom imunskom sistemu [76]. Hu i saradnici otkrili su da antioksidans MitoQ usmeren na mitohondrije smanjuje proizvodnju mitohondrijalnog ROS kroz aktivaciju Nrf2 puta, smanjuje oksidativni stres i oštećenje mtDNA i translokaciju fragmenata u citosol uzrokovanu oštećenjem tokom mezenterijalne I/R čime u celini štiti crevnu barijeru [77].

1.7.1.2. Signalni putevi za ublažavanje oksidativnog stresa

Nuklearni faktor 2 vezan za eritroide (*Nuclear factor erythroid 2-related factor 2,* Nrf-2) je ključni faktor transkripcije koji reguliše ćelijski antioksidativni odgovor na stres i utvrđeno je da ima ulogu u razvoju mnogih bolesti. U normalnim uslovima, Nrf2 vezuje specifični represorski protein Keap1 i formira kompleks Nrf2/Keap1, koji je lokalizovan u citoplazmi i bogat je cisteinskim ostacima. Kada nema stresa, poliubikvitinaciju (posttranslacijska modifikacija, to jest selektivna razgradnja proteina) vrši enzim Keap1/Cul3 ubikvitin ligaza i dolazi do brze razgradnje u proteazomima [78]. U odgovoru na oksidativni stres Nrf2 aktivira preko 200 citoprotektivnih gena, što pokazuje koliko je veliki značaj ovog faktora [79].

Kada telo reaguje na oksidativni stres, Nrf2 se odvaja od kompleksa Nrf2/Keap1 (kroz oksidaciju cisteina na specifičnim mestima Keap1), menja se konformacija, dolazi do kočenja ubikvitinacije Nrf2 i do njegovog ulaska u jedro, gde se dimerizuje sa malim muskuloaponeurotičnim fibrosarkomatoznim proteinom *(Musculoaponeurotic Fibrosarcoma*, *$_sMAF_s$*). U jedru se aktivirani Nrf2 vezuje za element antioksidativnog odgovora (*Antioxidant Response Element*, ARE), sekvence u promotorima ciljnih gena**.** Novoaktivirani Nrf2 pokreće ekspresiju gena za niz enzima, kao što su enzimi faze II detoksikacije, antioksidativni enzimi i enzimi uključeni u kontrolu glikemije [80,81]. Nrf2 na ovaj način dovodi do genske ekspresije (aktivacija ciljnih gena) i time povećava ekspresiju antioksidanasa kao što su hem-oksigenaza-1 (HO-1) i SOD, glutation S-transferaze (*Glutathione S-Transferase*, GST) i NADPH kinon oksidoreduktaza *(NAD(P)H:Quinone Oxydoreductase*, NQO1). SOD katalizuje razgradnju superoksidnih anjona (O_2^-) na O_2 i H_2O_2, koji se razlaže na H_2O i O_2 delovanjem katalaze. Takođe dolazi i do ekspresije antiapoptotičkih proteina Bcl2 i Bcl XL. U celini se smanjuje nivo ROS-a i štite se ćelije od oksidativnog stresa [82]. Slobodni hem je

izuzetno lipofilan i ubacuje se u ćelijsku membranu okolnih ćelija tkiva izloženog stresu, posledično aktivirajući vaskularne endotelne ćelije i dovodeći do povećanja adhezionih molekula(*Intercellular Adhaesion Molecule, ICAM-1, Vascular Adhaesion Molecule 1, VCAM 1*) i medijatora inflamacije [83].

HO-1 je enzim odgovoran za katabolizam hema, katalizujući razgradnju hema i proizvodnju ugljen monoksida (CO), bilirubina i gvožđa. Istraživanja su pokazala da bilirubin, kao i biliverdin (biliverdin se pretvara u bilirubin dejstvom bilverdin reduktaze) ima antioksidativne efekte i da je endogeni antioksidans [84,85]. Iako CO nije antioksidans, on efikasno inhibira oslobađanje inflamatornih medijatora, povećava odnos antiapoptotičkog faktora Bcl-2 prema proapoptotičkom faktoru Bax1 i povećava stopu preživljavanja kod eksperimentalnih životinja [86]. HO-1 igra važnu ulogu u povredama intestinalne I/R zbog svojih antioksidativnih efekata. Zbog svog antioksidativnog efekta, Nrf2 i njegovi ciljni geni smatraju se zaštitnicima tkiva, posebno kod crevnih bolesti, kod kojih ekspresija Nrf2 poboljšava mukoznu barijeru creva i smanjuje intestinalni inflamatorni odgovor kroz smanjenje lipidne peroksidacije i infiltracije neutrofila, ali pomaže i obnavljanje crevne mukoze [87-89].

Osim antioksidativnih i antiapoptotskih uloga, Nrf2 ima i imunomodulatornu ulogu što može da se koristi u prevenciji nekih oblika kancera i autoimunih bolesti [90]. Nrf2 se smatra najvažnijim regulatorom citoprotektivne adaptacije organizma na oksidativni stres. Liu i saradnici su otkrili da briostatin-1 pokazuje zaštitni efekat na crevo tokom I/R lezije inhibicijom oksidativnog stresa i inflamacije najvećim delom upravo preko Nrf2/HO-1 signalnog puta. Poznato je da oštećenje intestinalne barijere može da poveća agregaciju makrofaga koji dodatno oslobađaju ROS što dovodi do još većeg oštećenja barijere [91].

Upotreba antagonista receptora interleukina-1 (IL-1Ra) ne samo da efikasno inhibira ekspresiju inflamatornih faktora (IL-1β, IL-6 i TNF-α), već i značajno podstiče ekspresiju Nrf2, HO-1 i SOD, čime se smanjuje oštećenje crevnog tkiva [92,93]. Jačanje Nrf2 puta i promovisanje nuklearne translokacije Nrf2 moglo bi da pruži novi terapijski put za lečenje intestinalne I/R lezije.

1.7.1.3. Interakcija između Nrf2 i Nf-kB puteva

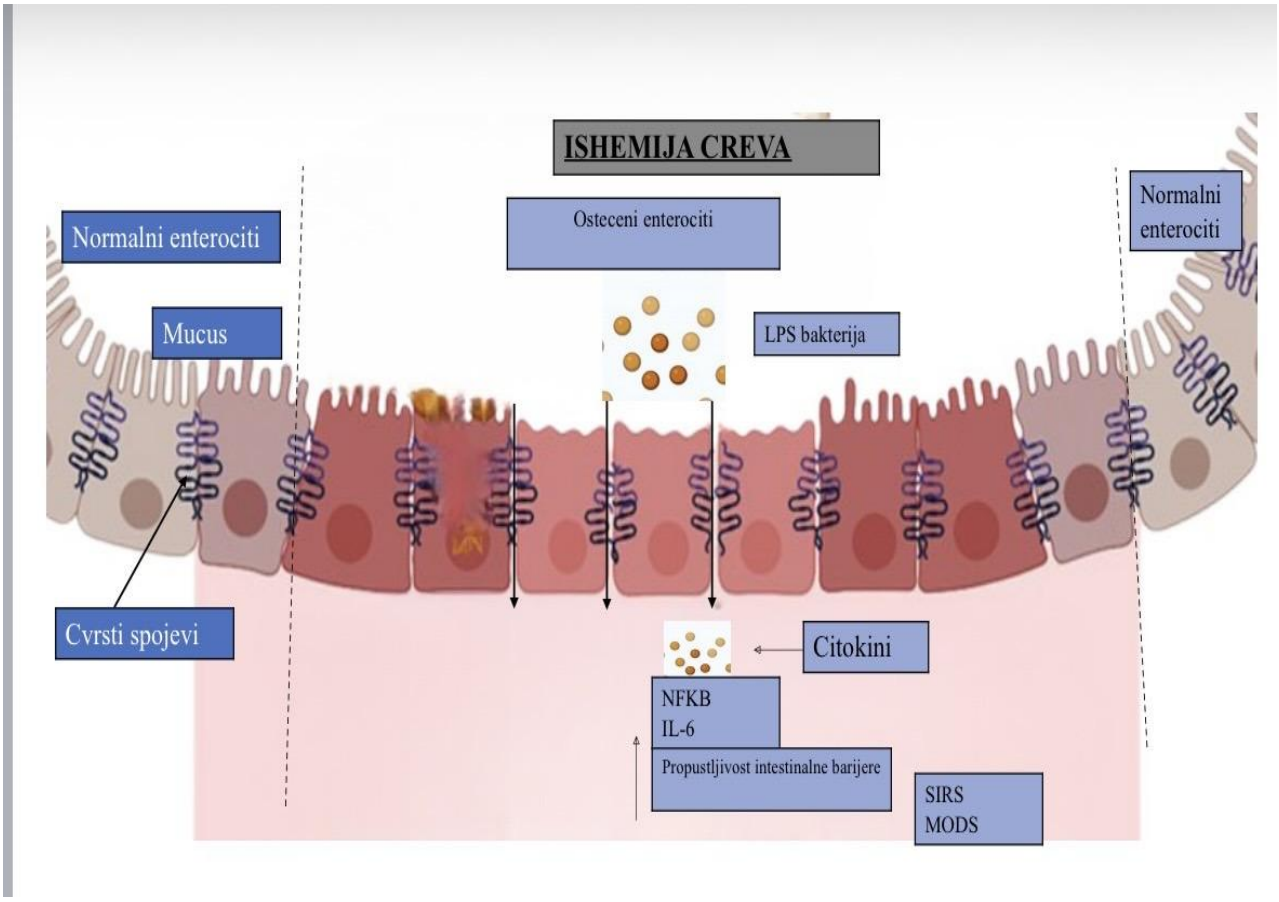
Ovi putevi su funkcionalno i najčešće recipročno povezani. Dok Nrf2 primarno stimuliše antioksidativne i citoprotektivne gene, NF-kB aktivira proinflamatorne gene. Njihova interakcija predstavlja ključnu tačku u kontroli ravnoteže između oksidativnog stresa i inflamacije [94,95]. Aktivacija Nrf2 može da koči NF-kB signalizaciju na više načina. Smanjenjem intracelularnih ROS i povećanjem ekspresije antioksidativnih enzima Nrf2 indirektno smanjuje aktivaciju IKK kompleksa a samim tim nema fosforilacije IkB i aktivacije NF-kB. Takođe, HO-1, u sklopu Nrf2 puta, može da koči nuklearnu translokaciju i transformaciju NF-kB putem svojih metaboličkih produkata kao što su ugljen monoksid i biliverdin [96,97]. Suprotno, dugotrajna aktivacija NF-kB može da suprimira Nrf2 funkciju povećanjem ekspresije Keap1 ili kompetitivnim vezivanjem za transkripcioni koaktivator CBP/p300, čime se smanjuje aktivnost ARE zavisne transkripcije [98]. U uslovima AMI disbalans između ova dva puta favorizuje inflamatorni odgovor preko oksidativnog oštećenja i povećanja produkcije citokina (TNF-alfa, IL-1beta, IL-6). Farmakološka modulacija koja povećava aktivnost Nrf2 i istovremeno suprimira NF-kB pokazala se efikasnom u eksperimentalnim modelima I/R lezija [99].

1.7.2. Inflamacija i infekcija u AMI

Mezenterijalna I/R lezija se razlikuje od sličnih I/R lezija na drugim organima upravo po bakterijskoj komponenti. Sve više publikacija tvrdi da su creva centralna u patofiziologiji razvoja kritičnog stanja kod AMI. Ovaj odnos je očigledan kada se kad se uzme u obzir visoka stopa sepse povezane sa AMI u poređenju sa drugim sindromima arterijske okluzije, kao što su infarkt miokarda ili moždani udar, što sugeriše na specifičnu septičku komponentu kod AMI [100, 101]. Jedna od najranijih zabeleženih veza između kritične ishemijske bolesti i creva jeste prisustvo bakterijskog endotoksina kod pacijenata u hemoragičnom šoku. Autori su saglasni sa hipotezom da je tokom perioda šoka telo jedinstveno ranjivo na translokaciju gram negativnih bakterija i njihovih endotoksina i pored primene antibiotika i intravenske nadoknade tečnosti [102].

Ovi izveštaji dali su povod za teoriju propuštanja creva, koje kada je oštećeno, omogućava bakterijsku translokaciju i sistemsku infekciju. Studija provedena na pacovima koji su bili podvrgnuti hemoragijskom šoku dala je dodatnu verodostojnost teoriji da šok fizički remeti normalnu funkciju barijere sluznice objašnjavajući kako šok rezultira bakterijskom translokacijom i endotoksemijom [103]. Novija literatura sugeriše da interakcija između crevnog epitela, mikrobiote i mukoznih imunoloških ćelija (rezidentni makrofagi) može objasniti patofiziološki uticaj I/R lezije. Jedna rana studija ispitivala je uticaj I/R povrede na funkciju barijere crevnog i endotelnog tkiva pacova merenjem protoka krvi i radioaktivno obeleženog albumina, kao i vizualizacijom tkiva putem elektronske mikroskopije. Utvrđeno je da je period ishemije sa naknadnom reperfuzijom u korelaciji sa stepenom lezije crevne barijere [104]. Studija u vezi s mehaničkom vezom između disfunkcije barijere i translokacije bakterija pokazala je da crevna inflamacija može da pokrene transćelijsku migraciju normalno neinvazivnih vrsta bakterija čak i pre prekida čvrstih spojeva između enterocita, za koje se dugo smatralo da su put kojim se bakterije kreću pored epitela, paracelularno [105]. Osim izazivanja disfunkcije barijere, I/R lezija značajno utiče na broj, kao i na fenotipove limfoidnih čvorova creva miševa analiziranih u eksperimentalnoj studiji [106]. Sama mikrobiota utiče i stupa u interakciju s različitim tkivima, posebno s onim crevnim uz koje se nalazi. Ovo može biti potvrđeno novijom literaturom koja pokazuje da je odgovor mišjih neutrofila na I/R leziju različit zavisno od bakterijske kolonizacije domaćina [107]. Ovu ideju podržava i nedavna studija koja je pokazala zaštitni efekat deksmedetomidina u ublažavanju intestinalne I/R lezije i poboljšanjem motiliteta creva modulacijom crevne flore [108]. Gore navedene studije zbirno predstavljaju snažan dokaz da se odgovor creva na I/R leziju može objasniti kompleksnim interakcijama između crevnog epitela, crevnog imunološkog sistema i crevnih endogenih, komensalnih bakterija. Eksperimenti na životinjama su ponudili uvid u potencijalnu ulogu koju antibiotici mogu imati u ublažavanju oštećenja povezanih s I/R lezijom u AMI. Pokazalo se da se značajan deo lezije u I/R odnosi na reperfuziju ishemijskog tkiva. Tokom trajanja reperfuzije ROS i drugi štetni inflamatorni citokini dodatno otežavaju ishemijsko oštećenje. Ispitivanje uticaja crevne mikrobiote na odgovor miša na I/R leziju otkrilo je da, dok su konvencionalni miševi imali izražene intestinalne i plućne upalne odgovore sa 100% smrtnošću, miševi bez mikroorganizama (*germ free animals*) nisu pokazali izraženu inflamaciju i nisu imali smrtnost u istim uslovima [109]. Sa biološkog stajališta, čak i kada je intestinalna vitalnost očuvana, funkcija epitelne barijere je ipak ugrožena I/R lezijom i dovodi do direktnog izlaganja bakterijama mukoze i submukoze, gde se nalazi veliki deo intestinalnih ćelija urođenog imunskog sistema (npr. makrofagi, dendritske stanice, limfociti)

i odvodni krvni sudovi sa inflamatornim ćelijama poput neutrofila, slika 5. Krvni sudovi iz crevnog zida se ulivaju u sistem vene porte i to je put propagacije ovog štetnog procesa. Takva direktna izloženost bakterijama normalno sterilnog prostora crevnog tkiva koji zauzimaju ćelije imunskog sistema neizbežno dovodi do značajne inflamacije. Antibiotska terapija pomaže u prevenciji kako ogromnog inflamatornog odgovora tako i bakterijemije [37]. Osim vazodilatatora i antibiotika u brojnim eksperimentima ispitivani su i razni farmakološki agensi koji deluju protektivno, najčešće antioksidativno, tokom I/R lezije u AMI.



Slika 5. Oštećenje intestinalne barijere (prekid čvrstih spojeva, *tigh junction*) tokom I/R lezije i translokacija bakterija. Dolazi do kontakta bakterija sa makrofagima u lamini propriji mukoze.

1.7.3. Apoptoza u AMI

Apoptoza se definiše kao programirana ćelijska smrt. Programirana ćelijska smrt je nešto širi pojam i pored apoptoze obuhvata još i piroptozu, feroptozu i autofagiju. Apoptoza se često razmatra i kao fiziološki proces. Ima ulogu u organogenezi, tokom rasta, imunoregulaciji, infekciji, uopšte u tkivnoj homeostazi. Organizam se ovim procesom oslobađa istrošenih, oštećenih ćelija, neželjenih i mutiranih ćelija. Mehanizam apoptoze je veoma složen i zapravo se sastoji od sleda molekularnih događaja koji rezultiraju smrću ćelije. Proces je koordinisan putem ekspresije gena, različitim signalnim putevima i sukcesivnom aktivacijom proteina [110]. U patofiziologiji I/R lezije ona dolazi na kraju, nakon oksidativnog stresa i inflamacije**.** Najizraženija je oko 1 sat nakon I/R lezije i smanjuje se tokom trajanja reperfuzije [111]. Ćelije izložene apoptozi pokazuju fragmentaciju jedarne DNK koja se smatra najspecifičnijim nalazom, kondenzaciju hromatina, smanjenje obima cele ćelije, stvaranje malih apoptotskih vakuola i apoptotskih tela [112].

Ključni enzimi apoptoze su kaspaze. Brojne biohemijske promene se dešavaju u apoptozi a glavna je aktivacija enzima kaspaza. Kaspaze su proteolitički enzimi i pripadaju porodici cistein endoproteaza. Za razliku od apoptoze, nekroza je akcidentalna, neprogramirana ćelijska smrt koja nastaje kao odgovor organizma na drastični stres, patološki podražaj [113,114]. Kaspaze se nalaze u svojim prekursorskim formama i aktiviraju se tokom apoptoze. Kaspaze se dele na inicijatorske i egzekutorske. Ovi značajni enzimi mogu da se aktiviraju na dva načina, unutrašnjim i spoljašnjim putem.

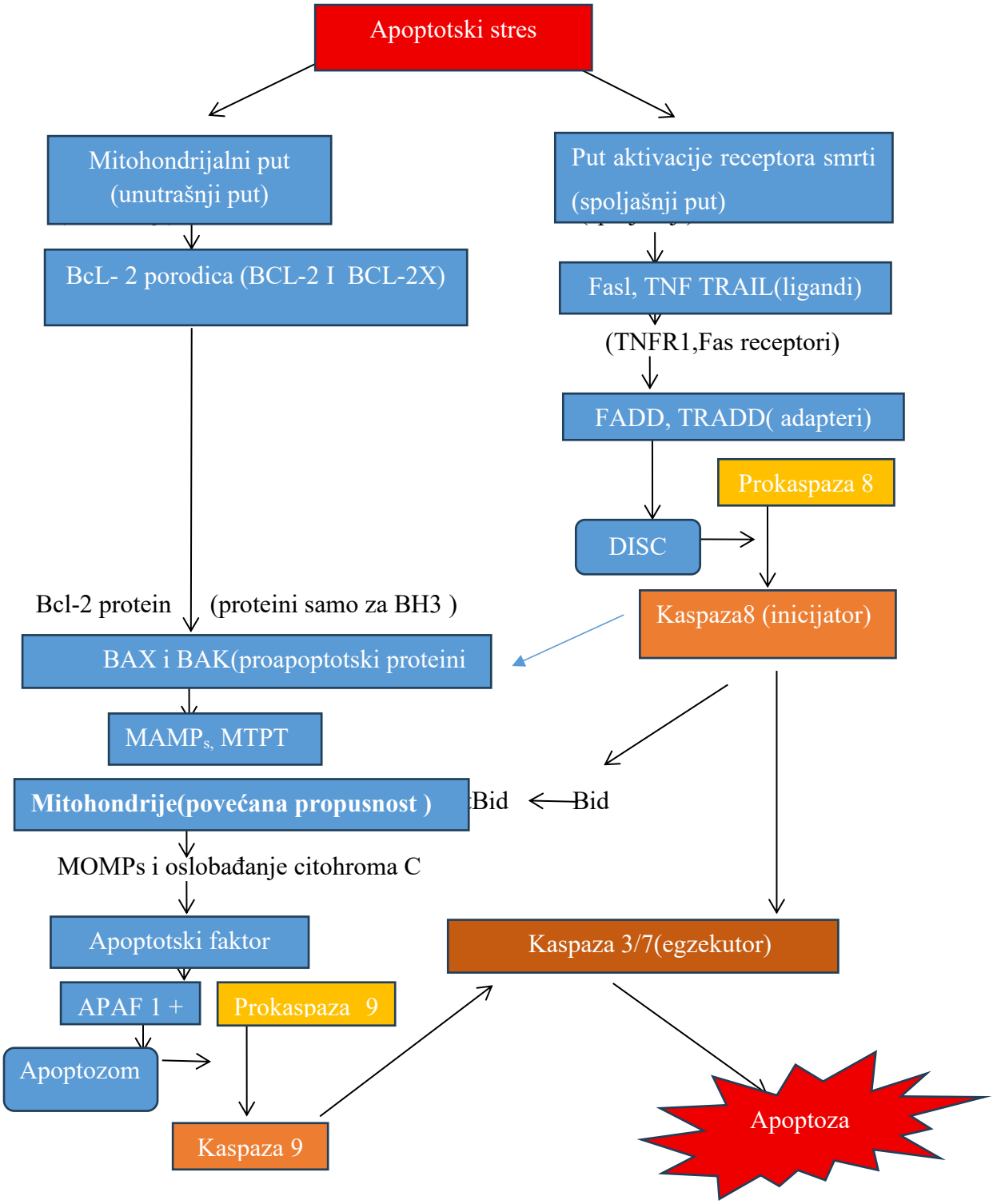
Unutrašnji put aktivacije dovodi do propusnosti membrane mitohondrija i oslobađanja proapoptotskih faktora u citosol, od kojih je najvažniji citohrom c. Dejstvom stresa ili drugim citotoksičnim uticajem dolazi do promena na familiji proteina Bcl-2 (*B cell lymphoma leukemia-2*). Ova grupa proteina ima proapoptotske i antiapoptotske članove. Najvažniji proapoptotski proteini ove grupe su Bax i Bak (*Bcl-2 Antagonist Killer*) koji se nalaze i neaktivnim oblicima u citosolu. Proteini samo za BH3 su takođe proapoptotski i interesantni su jer predstavljaju vezu između spoljašnjeg i unutrašnjeg puta aktivacije. Tokom apoptoze ova dva proteina se menjaju (konformacija i oligomerizacija) i deluju na membranu mitohondrija dovodeći do otvaranja specifičnih pora, tzv. mitohondrijalne tranzicione propusne pore (*Mitochondrial Permeability Transition Pore*, MPTP) putem molekularnih i metabolički povezanih molekularnih obrazaca, (*Microbe And Metabolism Associated*

Molecular Paterns, MAMPs). Ove pore su zavisne od jona kalcijuma i njihovo kratkotrajno otvaranje je korisno za ćeliju, dok je dugotrajno otvaranje štetno i citotoksično. Ukoliko otvaranje pora potraje duže, dolazi do povećane permeabilizacije spoljašnje membrane mitohondrija, *(Mitochondrial Outer Membrane Permeabilization,* MOMP) i oslobađa se citohrom c. Ovaj citohrom u sadejstvu sa faktorom aktivacije apoptotske proteaze i prekursorskom formom kaspaze 9 (prokaspazom) formira apoptozom. Na kraju, dejstvom apoptozoma na inicijatorsku kaspazu 9 dolazi do aktivacija egzekutorske kaspaze 3 (CC3) i do apoptoze [115-119].

Spoljašnji put aktivacije apoptoze je jednostavniji i dešava se preko receptora smrti. Glavni inicijator spoljašnjeg puta u mnogim tkvima jeste kaspaza 8 koja često direktno aktivira CC3 kao egzekutora. U nekim tkivima kao što je jetra, postoji takozvano pojačavanje apoptoze iz mitohondrija jer kaspaza 8 deluje na neaktivni citosolni BH3 preko BID-a, proapoptotskog člana familije proteina, koji aktiviran deluje na Bax i Bak (aktivacijom BAX i BAK gena) a dalje na isti način dolazi do dalje aktivacije kaspaze 9 i kaspaze 3 [120]**.** BID je pro-apoptotski član familije pro Bcl-2 proteina koji se aktivira kaspazom 8 u odgovoru na aktivaciju Fas/TNF-R1 receptora smrti. Aktivirani BID se kreće ka mitohodrijama gde indukuje oslobađanje citohroma c što dovodi do aktivacije egzekutorskih kaspaza i same apoptoze [121].

Kada dođe do aktivacije spoljašnjeg puta, ekstraćelijski TNF ligandi, Fas ligandi i ligand koji indukuje TNF-vezanu apoptozu (*TNF-related apoptosis-inducing ligand,* TRAIL), vezuju se za svoje receptore smrti u membrani i formiraju domene smrti- (*Fas Associated Death Domain*, FADD) i (*Tnf- Related Apoptosis Inducing Death Domain*, TRADD). Nakon formiranja domena dolazi do aktivacije kaspaze 8 preko signalnog kompleksa koji izaziva smrt (*Death-Inducing Signaling Complex,* DISC). Ova kaspaza potom direkno aktivira efektorske kaspaze 3 i 7. TRADD može da koči FADD vezivanje za receptore što može da dovede do smanjenja aktivnosti kaspaza. Ovo saznanje može da se iskoristi u onkologiji za lečenje raka takozvanim agonistima TRAIL receptora [122,123]. Mehanizmi apoptoze su prikazani na slici 6. Promene u mitohondrijama pojačavaju apoptozu kroz jaču inicijaciju ka egzekutorskim kaspazama. Na kraju apoptoze od ćelija ostaju apoptotska telašca *(apoptotic bodies*), koja fagociti brzo prepoznaju i eliminišu [124]. U nekim studijama apoptoza je maksimalna 70 minuta nakon reperfuzije. Apoptoza se pojavljuje i u normalnom crevu, ali kad nastupi ishemija dolazi do izraženog ćelijskog odumiranja. Inhibicija apoptoze je mehanizam zaštite creva koji poboljšava vitalnost i oporavak creva u eksperimentalnim

studijama sa okluzijom SMA. Ovaj pristup osim što štedi creva može da utiče i na SIRS koji često prati AMI [125,126].



Slika 6. Mehanizmi apoptoze. Uočava se konvergencija ova dva puta ka mitohondrijama i ka aktivaciji kaspaza 3 i 7 kao izvršiocima apoptoze.

Apoptoza stoji nasuprot mitoze u organizmu i neke od situacija gde je bitan odnos između ova dva procesa jesu autoimuni i neoplastični procesi i nastanak ožiljaka. Apoptoza u fiziološkim uslovima optimizacijom crevne strukture i broja ćelija osigurava odgovarajući razvoj i održavanje strukture i funkcije creva. Blokadom mitoze deluju neki antimitotički agensi (citostatici) i tada prevagu ima apoptoza [127,128].

Nelečena intestinalna I/R lezija dovodi do narušavanja intestinalne barijere i razvoja SIRS-a i MODS-a pri čemu apoptoza i nekroza imaju značajnu ulogu. Apoptoza se značajno pojavljuje u ishemijskom tkivu. Opisano je da epitelne ćelije tankog creva izložene I/R leziji u animalnim modelima pokazuju značajnu apoptozu. Tokom ishemije ćelije zahvaćenog tkiva creva nemaju dovoljno kiseonika i nutrijenata što dovodi do metaboličkih poremećaja i fragilnosti ćelija. Ovakve promene ubrzavaju proces apoptoze koja dodatno može da ubrza inflamaciju [129,130]. Za vreme postishemijske reperfuzije oštećenje crevnog tkiva raste usled metabolita generisanih tokom ishemije i oslobađanja većih količina ROS-a. U takvim okolnostima još većeg stresa, tkivo reaguje nekrozom, neprogramiranom i nekontrolisanom ćelijskom smrću. Iako još nije utvrđena direktna uzročno-posledična veza između apoptoze i nekroze u intestinalnoj I/R leziji, smatra se da postoji značajna interakcija između ova dva procesa [131-133].

Oksidativni stres, inflamacija i apoptoza čine međusobno povezanu patofiziološku trijadu tokom intestinalne I/R lezije. Radi se o kaskadi procesa u kojoj ima brojnih interakcija, u smislu stimulacije i inhibicije. ROS pojačavaju inflamaciju i apoptozu, inflamacija pojačava oksidativni stres i dodatno aktivira apoptotske signale. Apoptoza doprinosi gubitku epitelne crevne barijere i omogućava bakterijsku translokaciju, a može i dodatno da poveća infiltraciju makrofaga i neutrofila, čime se pojačava SIRS koji napreduje do MODS-a [37, 134-138].

1.8. Dijagnoza AMI

Široka je diferencijalna dijagnoza kod pacijenata sa akutno nastalim bolom u trbuhu. Nalazi fizikalnog pregleda i najčešće laboratorijske abnormalnosti pronađene kod AMI-a nisu dovoljno osetljive niti specifične za dijagnozu. Istraživači su još uvek u potrazi za idealnim biomarkerom AMI u plazmi [139]. Laktacidoza, koja se smatrala važnim laboratorijskim nalazom jer ukazuje na anaerobni metabolizam usled prevage laktata nad piruvatom i pada pH vrednosti, danas nije korisno sredstvo zbog svoje nespecifičnosti. Razvija se kasno u toku

AMI-a kada postoji ekstenzivni transmuralni infarkt i u tom trenutku već je jasno da postoji ozbiljan intraabdominalni proces ili abdominalna katastrofa i tada je mortalitet veći od 75% [140].

Stoga, multidetektorska CT angiografija (MDCTA) ima visoku specifičnost i osetljivost i trebalo bi da bude prva linija snimanja kod sumnje na AMI zbog svoje visoke dijagnostičke tačnosti [141,142]. Zbog nejasne kliničke slike i nespecifičnih laboratorijskih nalaza, nedovoljna klinička sumnja može dovesti do gubitka dragocenog vremena. Tokom ovog dijagnostičkog kašnjenja, progresija ishemije u transmuralni infarkt creva s peritonitisom i septikemijom može dodatno pogoršati ishode pacijenata. Jednostavan, brzi i neinvazivni biohemijski test bio bi idealan za povećanje kliničke sumnje na AMI i poboljšao bi odabir pacijenata za radiografsku evaluaciju. Eksperimentalne *in vitro* i *in vivo* studije pokazuju da citrulin, glutation S-transferaza (GST) i protein koji vezuje intestinalne masne kiseline (*Intestinal Faty Acid Binding Protein,* I-FABP) mogu da posluže kao rani markeri za AMI [143].

Pacijenti sa mezenteričnom ishemijom često imaju leukocitozu, metaboličku acidozu, povišen D-dimer i povišen nivo laktata u serumu. D-dimer se takođe smatra važnim alatom u dijagnostici AMI, jer normalna vrednost isključuje AMI. Međutim, D-dimer, enzimski produkt razgradnje fibrina koji se oslobađa tokom intravaskularne koagulacije i taloženja fibrina, može biti prisutan kod AMI kao i kod nekoliko drugih stanja. Nedavna studija Thuijlsa i saradnika potvrdila je da se nivo L-laktata u plazmi, manjak baze (niži pH) i broj leukocita ne mogu koristiti kao markeri za AMI [144]. Klasično opisani laboratorijski nalazi (SE, CRP, prokalcitonin, KS, lipaza, amilaze, transaminaze, urea, kreatinin) takođe se ne mogu koristiti kao markeri za ranu dijagnozu AMI [145]. Ovi laboratorijski nalazi nisu dovoljno osetljivi ili specifični da bi se ustanovila ili isključila dijagnoza AMI. Porast serumskih markera tokom AMI obično se javlja tek nakon transmuralnog infarkta creva i stoga se ne mogu rutinski koristiti za ranu dijagnozu [146]. Kod AMI, ishemija počinje na sluznici i proteže se prema serozi, što pruža vremena da se reaguje dijagnostički i terapijski [147]. Idealan biomarker za mezenteričnu ishemiju trebalo bi da nastane na sluznici kako bi se ishemija otkrila u najranijoj fazi. Memet i saradnici ističu da novi serumski biomarkeri ukazuju na disfunkciju intestinalne barijere u ranoj fazi. Pored citrulina, I-FABP i GST to su i D laktat, ishemijom modifikovani albumin, TBARS i tkivni malondialdehid (MDA) kao indikatori lipidne peroksidacije [148].

1.9. Terapijski pristup AMI

U terapiji AMI neophodan je multidiscipinarni pristup, a tim treba da čine hirurg, vaskularni hirurg, intenzivista, interventni radiolog i kardiolog. Početna terapija treba uključivati izdašnu intravensku nadoknadu tečnosti, (1-2 ml ringer laktata/ kg telesne težine/ sat), satna diureza ne sme da bude ispod 50 ml, dekompresiju nazogastričnom sondom i, ako je moguće, direktne antikoagulanse (*Direct Oral Anticoagulance,* DOAC*)* i inhibitore K vitamina*(Vitamin K Antagonists,* VKA), važno je izbegavanje vazokonstriktorskih sredstava koja mogu da pogoršaju situaciju u vaskularnom koritu [20,33]. Antibiotike širokog spektra treba davati rano shodno vodičima za tretman AMI [20,149,150]. Antikoagulansi su primarna terapija u slučajevima MVT, potencijalno uz obaveznu dugotrajnu antikoagulantnu terapiju ako se otkrije primarni poremećaj zgrušavanja.

Za bilo koji uzrok AMI, laparotomija je indikovana ako postoje peritonealni znaci. Kod većine pacijenata obično je neophodna hitna hirurška embolektomija ili bajpas, sa resekcijom nekrotičnog dela creva. Imajući u vidu da nije moguće precizno predvideti vitalnost creva ishemijskog izgleda, revaskularizacija bi trebalo da prethodi resekciji, jer obnavljanje protoka u SMA može dovesti do značajnih promena u segmentima creva koji su u početku izgledali ireverzibilno ishemijski. Glavni princip lečenja je obnavljanje dotoka krvi u ishemijsko crevo i resekcija nekrotičnog segmenta. Rana revaskularizacija je važna za smanjenje mortaliteta [149].

Zbrinjavanje pacijenata sa AMI je i dalje izazovno. Hirurgija i endovaskularne intervencije su dva glavna pristupa lečenju pacijenata s mezenteričnom ishemijom. Međutim, i sama reperfuzija doprinosi leziji creva jer tada nastaje oslobađanje većih količina ROS i RNS. Kod pacijenata u šoku, uklanjanje nekrotičnog creva je prioritet jer se sumnja na sepsu. Kod pacijenata bez sumnje na intestinalnu nekrozu, obnova perfuzije je prioritet kako bi se izbegla crevna nekroza. Sa ili bez resekcije creva, treba razmotriti laparotomiju drugog pregleda kako bi se proverila crevna vitalnost 24 do 48 h nakon prve operacije. Hirurgija kao modalitet lečenja pacijenata sa embolijom i trombozom mezenterične arterije ima tri cilja, uključujući: (1) uklanjanje tromba ili embolusa, (2) lečenje osnovne stenoze angioplastikom ili bajpasom, i (3) resekcija nekrotičnog creva sa stomom, bez izvođenja anastomoze, sa kreiranjem

ileostome ili kolostome, prema principima hirurgije kontrole oštećenja (*Damage Control Surgery*, DCS) [150,151].

Nakon što je dijagnoza AMI evidentna ili postoji velika sumnja na osnovu CT-a i kliničkih nalaza, sledeći korak je proceniti da li je pacijentu potrebna laparotomija da bi se procenila vitalnost creva i kontrola oštećenja ili da li se pacijent može podvrgnuti pokušaju endovaskularne revaskularizacije SMA bez početne laparotomije. Dijagnoza transmuralne nekroze creva i procena potrebe za laparotomijom jedna je od najvažnijih odluka koje hirurg mora doneti. Nedavna meta-analiza identifikovala je nekoliko kliničkih karakteristika, mozaik promena, kao što su insuficijencija organa, SIRS, dugotrajnost simptoma, koronarna arterijska bolest i šok, biohemijski markeri kao što su: povišeni serumski laktat, acidoza, leukocitoza, hemokoncentracija, hiperamilazemija i CT radiološki nalazi- proširenje crevnih vijuga, pneumatoza, arterijska ili venska mezenterična okluzija, slobodna intraperitonealna tečnost koje u kombinaciji mogu ukazivati na povećan rizik od nekroze creva [152].

Terapijski modaliteti za sada nisu obećavajući. Visoka stopa mortaliteta AMI perzistira i dalje, a bolest ima rapidni, progresivni tok koji za 8-12 sati dovodi do SIRS-a i MODS-a [7]. To je razlog zašto nema kliničkih studija koje bi analizirale terapijske mogućnosti. Poslednjih godina su iz istog razloga razvijeni brojni eksperimentalni modeli u kojima se pokušava rani farmakološki pristup na eksperimentalnim životinjama koji bi popravio ishode lečenja. Primenjeno je puno supstanci, najčešće antioksidansi, sa obećavajućim rezultatima, ali još uvek nema kliničke primene [153].

1.10. Eksperimentalni modeli AMI

Alternativa hirurškom pristupu pre nastanka ireverzibilnih promena su antikoagulansi, trombolitici , vazoaktivni lekovi, opšta suportivna terapija (nadoknada tečnosti i elektrolita, resuscitacija). Prema Fuglsethu na vreme primenjeni antikoagulansi (DOAC i VKA), i u optimalnoj dozi, omogućavaju rekanalizaciju u više od polovine slučajeva [154]. Razumevanje patofizioloških procesa i kaskade koja se dešava tokom ishemije i reperfuzije može doprineti razvoju novih terapijskih strategija. Istraživačka terapija AMI podrazumeva farmakološki pristup u ranoj fazi i poslednjih desetak godina se pojavilo mnoštvo supstanci koje su ispitivane na animalnim modelima sa često obećavajućim rezultatima [155]. Postoji i nefarmakološki pristup kao što je pre i postkondicioniranje koje kroz ponavljane kratkotrajne

ishemije i reperfuzije čine ciljano tkivo creva i udaljene organe otpornijim na intestinalnu ishemijsko-reperfuzijsku povredu [156].

Najviše studija je rađeno sa Wistar pacovima muškog pola, a nekoliko studija je rađeno na pacovima ženskog pola. Starost životinja je bila od 6 do 18 nedelja, najčešće 8-12 nedelja, težine od 200 do 350 gr. Najčešće korišćen i najbolje opisan je bio model okluzije gornje mezenterične arterije kod Wistar pacova. Nakon pažljive preparacije krvnog suda od okolnog tkiva postavlja se klema a uspešna okluzija se makroskopski uočava prestankom pulsacija i bledilom a potom i lividitetom crevnih vijuga. Ovaj model je korišćen u ovoj studiji. Za okluziju se koristi atraumatska mikroklema iz razloga da ne dođe do mehaničke lezije endotela i da bi se kasnija reperfuzija adekvatno evaluirala. Vreme trajanja okluzije se kreće od 30 minuta do 90 minuta. Reperfuzija se najčešće sprovodi do 120 minuta i započinje pažljivim skidanjem atraumatske kleme sa SMA [157,158]. Reperfuzija se poznaje po pulsacijama u krvnim sudovima mezenterijuma, vraćanju normalnog kolorita creva i po pojavi peristaltičnih talasa.

Osim kompletne okluzije SMA atraumatskom klemom, rađeni su i ogledi sa kompletnom vaskularnom okluzijom, klemovanje i arterije i vene mezenterike superior. Osim klemovanja, rađena je i ligatura SMA ali tada, naravno, nema mogućnosti da se ispituje reperfuziona lezija, već samo ishemija. Koristi se i takozvani "Porcine"model perkutane ili endovaskularne embolizacije SMA butil 2 cijanoakrilatom ili polivinil alkoholom. Ovaj model je najbliži kliničkom dešavanju tokom akutne mezenterijalne ishemije jer nema laparotomije niti manipulacije crevima, okluzija protoka se ne vrši pritiskom spolja nego opstrukcijom protoka, dakle iz lumena krvnog suda kao što to čini embolus ili tromb [157,158]. Opisani su radovi gde je primenjena segmentna vaskularna okluzija pri čemu su klemovane pojedinačne grane SMA, što je poznato kao Murine model [159]. Najčešće su mereni parametri oksidativnog stresa, markeri inflamacije i endotelne disfunkcije. Ispitivani su enzimi jetre, vrednosti ureje i kreatinina, parametri u BALF-u, sve u cilju otkrivanja lezija udaljenih organa, kao i imunohistohemijske i patohistološke analize. Primena farmakoloških agenasa je u eksperimentima bila najčešće kao pretretman, pre izazivanja I/R lezije [160]. Mnogi agensi su primenjivani u fazi ishemije i početkom reperfuzije. Tako je i levosimendan primenjen u postreperfuzionom periodu [161,162]. Smatra se da lezije udaljenih organa najviše nastaju tokom rane faze reperfuzije i kasnije tokom 1 do 6 sati i to signalnim putem NF-kB i TLR [163]. Najčešće i najbrže je pogođena jetra putem portne cirkulacije [164]. Imajući u vidu patofiziologiju AMI koja se sastoji od serije povezanih procesa kao što su vazokonstrikcija,

tromboza, oštećenja mitohondrija, inflamatorni odgovor i ćelijska smrt, razvijaju se različiti mehanizmi zaštite [165]. Farmakološki agensi tokom akutne mezenterijalne ishemije i kasnije reperfuzijske povrede deluju na sledeće načine: antioksidativno, antiinflamatorno, kroz jačanje ćelijske membrane, i povećanjem energetskih potencijala same ćelije dejstvom na mitohondrije.

Adekvatnost ovog modela je potvrđena u smislu trajanja ishemije i reperfuzije, i to 30 minuta ishemije i 90 minuta reperfuzije. Poslednjih godina se teži da se ishemija produži na 60 minuta a reperfuzija i na više od 120 minuta [135,166]. Adekvatnost primenjene doze levosimendana je u skladu sa literaturom gde se nailazi na doze od 1mg/kg, 2 mg/kg i 4 mg/kg, zavisno od ciljeva istraživanja [162,167].

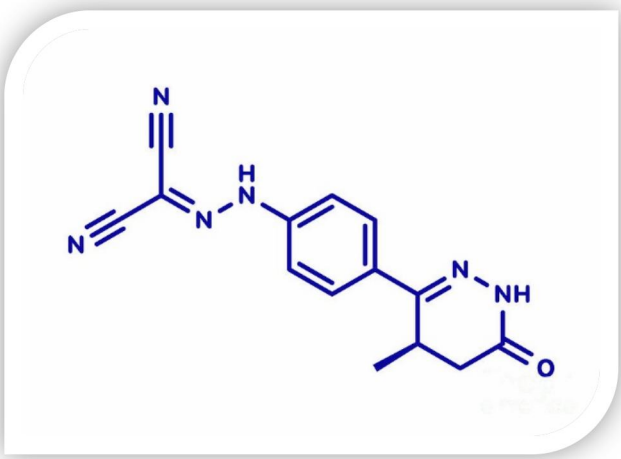
1.11. Levosimendan

Kardiotonični lekovi uglavnom uključuju digitalis, kateholamine, inhibitore fosfodiesteraze i kalcijumske senzibilizatore. Digitalis, kateholamini i inhibitori fosfodiesteraze povećavaju kontraktilnost miokarda povećanjem intracelularnih koncentracija cikličkog adenozin monofosfata (cAMP) i Ca^{2+}, a ovo povećanje intracelularne koncentracije kalcijumovih jona povećava potrošnju kiseonika u miokardu i uzrokuje aritmiju. Iz ovih razloga, fokus istraživanja na pozitivnim inotropnim agensima se pomerio sa mobilizacije kalcijuma na senzibilizaciju kalcijuma. Intracelularni senzibilizatori kalcijuma su efikasniji i sigurniji lekovi jer ne povećavaju unutarćelijsku koncentraciju jona kalcijuma. Jedan od ovih lekova, levosimendan, ima višestruke molekularne ciljeve i ispoljava svoje farmakološke učinke ne samo povećanjem kontraktilnosti miokarda, već i jačanjem funkcije respiratornih mišića, zaštitom creva, jetre i bubrega, a koristan je i za pacijente s teškom sepsom i septičkim šokom. Levosimendan ima dakle, pored inodilatatornog dejstva i antiinflamatorno, antioksidativno i antiapoptotsko dejstvo (plejotropni efekti).

1.11.1. Struktura i mehanizam dejstva

Levosimendan pripada grupi aktivnih sastojaka koji se nazivaju senzibilizatori kalcijuma i ima pozitivan inotropni učinak povećavajući osetljivost srčanih miofilamenata na jone kalcijuma. Senzibilizatori kalcijuma su lekovi koji pri istoj koncentraciji kalcijuma, dovode do većeg razvoja sile u pojedinačnim mišićnim poprečnim vlaknima bez povećanja potrošnje

energije mišićne ćelije [168]. Levosimendan je opisan kao kalcijum senzibilizator racionalno dizajniran i testiran da deluje kroz njegovo vezanje zavisno od kalcijuma za srčani troponin C [169]. Levosimendan je bio prvoklasno sredstvo u vreme svog nastanka, promovišući uglavnom inotropiju kroz kalcijumsku senzibilizaciju srčanog troponina C (cTnC). Levosimendan, kako su izvestili Pollesello i dr. 1994., vezuje se za ljudski cTnC zasićen kalcijumom [169]. Dihidrodipiridazonski prsten je odgovoran za vezivanje levosimendana za TnC i senzibilizaciju kalcijuma [170]. Formula levosimendana je C14H12N60, slika7.



Slika 7. Struktura levosimendana.

Za levosimendan je u toku razvojnog programa utvrđeno da posreduje u otvaranju ATP-zavisnih kalijumskih kanala (K_{ATP} kanala) u vaskularnim glatkim mišićnim ćelijama u različitim vaskularnim odeljcima [171]. Ovim mehanizmom delovanja, levosimendan izaziva povećanje perfuzije krvi u ključnim organima i sistemsku vazodilataciju kada se koristi u dozama unutar priznatog terapijskog raspona, što znači da se lek mora uzeti u obzir i koristiti kao inodilatator, a ne samo kao inotrop. Suštinski aspekt farmakologije i kliničkog profila levosimendana je da su njegovo poboljšanje perfuzije i sistemski vazodilatacijski efekti posredovani različitim mehanizmima i stoga mogu biti odvojeni jedan od drugog. Levosimendan, koji deluje na K_{ATP} kanale ima različit regionalni efekat u odnosu na sistemski efekat u poređenju sa lekovima kao što su PDE inhibitori (milrinon) [172]. Poseban naglasak mora se staviti na otkriće da levosimendan takođe otvara K_{ATP} kanale na unutrašnjoj membrani mitohondrija čime je delovao antiishemijski tokom I/R lezije miokarda [173,174].

Ovaj efekat je povezan sa kardioprotekcijom, smanjenjem veličine infarkta i ublažavanjem I/R povreda u studijama *in vitro*, *ex vivo* i *in vivo* na animalnim modelima [175,176].

Levosimendan je pokazao efekat kao pretretman i u kliničkim studijama, nakon aorto-koronarnog bajpasa [177]. Gore spomenuti efekti koji proizilaze iz senzibilizacije na kalcijum i vazodilatacije su zajednički sa dugodelujućim metabolitom levosimendana OR-1896, koji se formira u crevima putem redukcijsko-acetilacionog puta. Slobodne koncentracije leka i metabolita u plazmi su slične, ali klinički značajne koncentracije farmakološki aktivnog OR-1896 u plazmi se mogu detektovati danima nakon infuzije levosimendana (70-80 h) i doprinose postojanju terapijskog učinka nakon primene matičnog leka [178-180].

Osim ovih primarnih mehanizama, identifikovano je da levosimendan ima niz pomoćnih delovanja koji se opisuju kao plejotropni efekti, koja ne uključuju poboljšanje srčane funkcije, ali koja mogu biti implicirana u nekim kliničkim efektima i odgovorima na levosimendan [181]. Ovi efekti obuhvataju antiinflamatorno, antioksidativno i antiapoptotsko delovanje koje se može ispoljiti i u drugim organima, pored srca, uključujući bubrege, jetru, creva, pluća i dijafragmu i pomoćne respiratorne mišiće. Al Čalabi i saradnici naglašavaju da levosimendan svojom akcijom preko glatkih mišićnih ćelija u vaskularnom koritu i preko mitohondrija postiže bolju perifernu perfuziju, povećava glomerularnu filtraciju, povećava koronarni protok, uopšte ispoljava antiishemijski efekat i dovodi ne samo do kardioprotekcije već i do zaštite drugih organa, mada postoje dejstva koja još uvek nisu u potpunosti razjašnjena, što se naročito odnosi na inotropiju [182].

1.11.2. Levosimendan u kliničkoj upotrebi

Levosimendan je u kliničkoj praksi indikovan kod kardioloških pacijenata sa srčanom insuficijencijom, kao i kod pacijenata koji čekaju transplantaciju srca, Takotsubo sindrom, kardiohirurški pacijenti sa slabom ejekcijskom frakcijom leve komore i razvojem kardiogenog šoka. Veliko iskustvo sa levosimendanom stečeno je u manjim, često jednocentričnim, studijama. Mnoge od tih studija ukazuju na korist od levosimendana za preživljavanje, što je nalaz potvrđen i u meta-analizi [183]. Levosimendan je bio povezan sa povoljnim uticajem na razmatrane ishode. Ključne terapijske oblasti koje su praćene su obuhvatale kardiogeni šok, uznapredovalu srčanu insuficijenciju, kardiohirurgiju i sepsu, i kod svih je bilo koristi od terapije levosimendanom. Afirmativni nalazi ovih analiza mogu se uporediti sa sličnim efektima dobutamina i PDE inhibitora, koji su povezani sa ukupno lošijom srednjoročnom i dugoročnom prognozom. Ovi suprotni nalazi naglašavaju razliku između inotropa koji deluju,

bilo putem adrenergičkih ili PDE-ciljanih puteva, na povećanje unutarćelijskih nivoa cAMP u kardiomiocitima i levosimendana koji poboljšava kontraktilnost srca bez ugrožavanja dugoročne vitalnosti ćelija srčanog mišića [184,185].

Levosimendan ima brz, povoljan i dugotrajan uticaj na hemodinamiku i simptome kod akutne dekompenzovane srčane insuficijencije (*Acute hearth failure*, AHF). Složeni način delovanja levosimendana, sa pluralitetom efekata se pojavio i kao važan aspekt kliničke svestranosti i korisnosti leka i kao stimulans za medicinska istraživanja [186-188].

Vazodilatatorska dimenzija farmakologije levosimendana je relevantna za upotrebu leka u stanjima niskog protoka kao što je AHF, u kojoj je ključna patologija hipoperfuzije organa. Može se očekivati da će lek koji istovremeno povećava minutni volumen srca i poboljšava vazodilataciju u nekim slučajevima imati povoljniji učinak od leka koji deluje samo na minutni volumen srca [189]. Važnost korekcije neadekvatnog volumena pre početka terapije vazodilatatornom ili inotropnom terapijom se mora uzeti u obzir i pratiti tokom terapije, što je bitno i u eksperimentalnim istraživanjima. Levosimendan je pokazao efikasnost u stanjima kardiogenog šoka, kod stresne kardiomiopatije, Takotsubo sindrom, u kardiohirurgiji i akutnoj desnostranoj srčanoj insuficijenciji [190].

1.11.3. Istraživanja i razvoj izvan kardiologije

Levosimendan je proučavan na nesrčanim mišićima, posebno na respiratornim mišićima. *In vitro* studije su pokazale da levosimendan poboljšava osetljivost na jone kalcijuma i stvaranje sile u vlaknima dijafragme kod zdravih osoba i pacijenata s HOBP. U jednoj kliničkoj studiji levosimendan je u korištenoj dozi poboljšao kontraktilnu efikasnost dijafragme za 21% [191]. Fiziološko i farmakološko obrazloženje za primenu levosimendana kod amiotrofične lateralne skleroze (ALS) počiva na činjenici da i dijafragma i skeletni mišići eksprimiraju gene za sporu izoformu TnC, budući da se dijafragma sastoji od približno 50% vlakana koja se sporo kontrakuju [192]. Kao senzibilizator kalcijuma sa cTnC kao molekularnom metom, levosimendan tako može ojačati kontraktilnost i u dijafragmi i skeletnim mišićima. Višestruka farmakologija levosimendana može takođe pružiti dodatni klinički uticaj kod pacijenata sa ALS-om kroz već pomenuti niz farmakoloških efekata koji nisu direktno povezani sa delovanjem leka na senzibilizaciju kalcijuma [183].

Nedavna istraživanja o učinku levosimendana na oksidativni stres na mišjem modelu dijabetesa koja pokazuju učinak leka u prevenciji oštećenja pamćenja otvorila su novi mogući

razvojni put [193,194]. Eksperimentalna studija na pacovima, naglasila je zaštitne efekte izazvane levosimendanom protiv I/R lezije jetre [195,196]. Interesantno, novi farmaceutski agensi se razvijaju sa levosimendanom i cTnC kao farmakofornim modelom u neočekivanim poljima kao što je onkologija. Levosimendan zbog svojih brojnih dejstava kao što su inhibicija fosfodiesteraze 3, smanjenja produkcija NO preko inhbicije aktivnosti iNOS i smanjenje ROS-a, može da bude efikasan u onkološkoj terapiji. Levosimendan inhibira migraciju ćelija raka i senzibiliše hipoksične ćelije na zračenje, dok istovremeno štiti organe aktiviranjem mitohondrijskih kalijumskih kanala. Kada se levosimendan primeni istovremeno sa 5-fluorouracilom (5-FU) pokazuje sinergistički efekat na karcinom mokraćne bešike [197].

1.11.4. Multiorgansko delovanje levosimendana u kritičnim bolestima

Levosimendan uzrokuje vazodilataciju svojim delovanjem na K+ kanale u visokim koncentracijama u plazmi. Osim u miokardu, levosimendan izaziva vazodilataciju i u drugim organima, uključujući pluća, mezenterijum, jetru i bubrege [198]. Kao rezultat toga, perfuzija organa se poboljšava uprkos blagom padu krvnog pritiska. Kliničke posledice poboljšanja perfuzije tkiva povezane sa levosimendanom treba proceniti uzimajući u obzir istovremeno poboljšanje minutnog volumena srca. Pored svojih inotropnih i vazodilatatornih efekata, levosimendan ima nekoliko drugih važnih efekata, uključujući povećanje kontraktilnosti dijafragme [199], antiinflamatorno dejstvo [200-202], antiapoptotski efekat [203], utiče na funkciju trombocita, što dodatno pojačava njegov antiishemijski efekat [204,205]. Osim toga, levosimendan inhibira ekspresiju iNOS i proizvodnju azotnog oksida u ishemičnom tkivu [206, 207].

1.11.4.1. Levosimendan i funkcija respiratornih mišića

Studija van Hesa je pokazala je da intravenska injekcija levosimendana pojačava kontrakciju mišićnih vlakana dijafragme povećanjem intracelularne osetljivosti na kalcijum, što pruža snažnu teoretsku podršku za lečenje senzibilizatorom kalcijuma kod pacijenata s disfunkcijom respiratornih mišića praćenom HOBP [191]. Studija Schellekensa i saradnika je pokazala da levosimendan smanjuje markere oksidativnog stresa ali da ne utiče na inflamaciju dijafragme uzrokovanu mehaničkom ventilacijom i endotoksemijom na mišjem modelu [208].

1.11.4.2. Levosimendan i funkcije jetre i bubrega

Još uvek je vrlo malo studija o učincima levosimendana na funkcije jetre i bubrega. Postojeći izveštaji su pokazali da levosimendan može imati zaštitni učinak na funkcije jetre i bubrega. Studija Oktara i dr. u modelu insuficijencije jetre kod pacova, izazvane akutnom srčanom insuficijencijom, pokazala je da levosimendan sprečava I/R leziju jetre što je zapaženo putem histoloških pregleda i specifične imunohistohemije [209].

Studije Onodija i Grossinija pokazale su da levosimendan sprečava jetrenu I/R povredu kod pacova nakon embolizacije portalne vene, a specifični mehanizam može biti povezan s mitohondrijalnim K_{ATP} kanalima. Dakle, pretretman levosimendanom kod pacijenata sa parcijalnom resekcijom jetre može imati određeni zaštitni efekat na jetru [195,210]. Studija Brunnera i dr. je pokazala da levosimendan smanjuje apoptozu u ljudskim hepatocitima nakon I/R lezije [211]. Fedele i dr. ističu da levosimendan može imati potencijalne koristi za pacijente s akutnim srčanim i bubrežnim sindromima praćenim hipotenzijom [212]. Evropsko udruženje za srce preporučuje upotrebu levosimendana kod pacijenata sa srčanom insuficijencijom praćenom bubrežnom insuficijencijom kako bi se antiishemijskim dejstvom levosimendana i povećanjem bubrežne perfuzije krvi poboljšala i prognoza pacijenata [213]. Nekoliko studija na životinjama pokazalo je da levosimendan osim što poboljšava srčanu funkciju povećava i bubrežni protok krvi perifernim dejstvom na renalne arterije [214-216].

1.11.4.3. Levosimendan i sepsa

Veliki broj studija je pokazao da pluća, bubrezi, srce i skeletni mišići mogu biti oštećeni sepsom. Upotreba levosimendana kod teške sepse i septičkog šoka je vruća tema istraživanja kod pacijenata sa kritičnim bolestima. Dostupno je više randomiziranih kontrolisanih studija ove vrste sa pozitivnim rezultatima i u kojima je levosimendan smanjio smrtnost tokom sepse [217-223].

1.11.4.4 Levosimendan i AMI

Publikacije o dejstvu levosimendana na AMI se uglavnom odnose na eksperimentalne modele. U nekim radovima se radilo klemovanje SMA, a u nekim se izazivalo septično stanje, koje prati I/R leziju, obično ligacijom i punkcijom cekuma. Praćeni su biohemijski efekti levosimendana, kao što su antiinflamatorni, antioksidativni i patohistološki efekti na organe. Biohemijski je pokazan porast antiinflamatornih citokima i antioksidativnih jedinjenja, dok su

patohistološki potvrđeni protektivni efekti na crevo, jetru i bubrege [161,201,224-226]. Na osnovu svega izloženog, za očekivati je da ovakav agens ima povoljan, zaštitni uticaj na crevo i tokom eksperimentalno izazvane AMI.

2. CILJEVI ISTRAŽIVANJA

Cilj istraživanja ove disertacije je da se ispita potencijalno protektivno dejstvo levosimendana u pretretmanu akutne mezenterijalne ishemije na parametre oksidativnog stresa, inflamacije i apoptoze terminalnog ileuma na modelu I/R mezenterične arterije pacova. U realizaciji ovog cilja posebno će se pratiti efekti pretretmana levosimendanom na sledeće parametre:

1. Prooksidativni markeri (TBARS, O_2^-, H_2O_2 i $NO_2^{-)}$ u lizatu eritrocita i homogenatu terminalnog ileuma.
2. Markeri antioksidativne aktivnosti (SOD, CAT, GSH) u lizatu eritrocita i homogenatu terminalnog ileuma.
3. Prooksidativni markeri (TBARS i NO_2^-) u bronhoalveolarnom lavažu(BALF).
4. Markeri antioksidativne aktivnosti (SOD, CAT, GSH) u BALF-u.
5. Patohistološke promene na terminalnom ileumu, Chiu skor i gustina goblet ćelija.
6. Faktori inflamacije u crevu tokom ishemije i reperfuzije (CD 68 i IL-6).
7. Ekspresija proapoptotskih markera u terminalnom ileumu (NF-kB i CC3).

   kao i merenje TUNEL pozitivnih epitelijalnih ćelija tkiva tankog creva.
8. Ekspresija antiapoptotskih markera u terminalnom ileumu (Nrf-2 i HO-1).
9. Patohistološke promene na udaljenim organima kao što su srce, pluća i bubrezi.
10. Relativna genska ekspresija markera antioksidativne aktivnosti (SOD i CAT) u terminalnom ileumu pacova.

3. RADNE HIPOTEZE

U razradi ove studije definisali smo sledeće radne hipoteze:

1. Levosimendan primenjen kao pretretman akutne ishemije i reperfuzije mezenterijuma smanjuje produkciju markera oksidativnog stresa i markera inflamacije.
2. Levosimendan smanjuje leziju creva delujući antioksidativno, antiinflamatorno i antiapoptotski.
3. Levosimendan smanjuje lezije udaljenih organa (srce, pluća i bubrezi).
4. Levosimendan ima protektivni efekat u akutnoj mezenterijalnoj ishemiji.

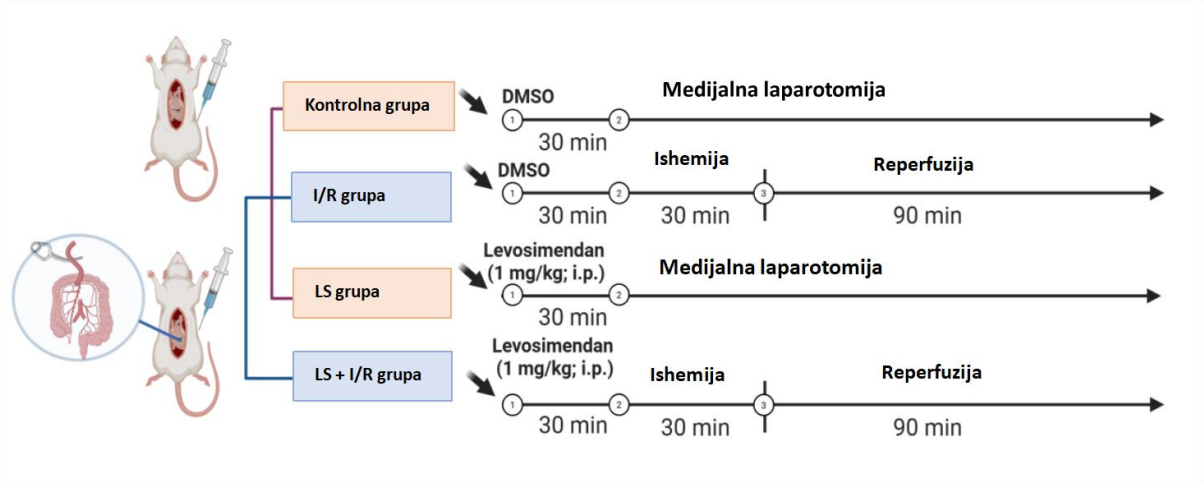
4. MATERIJAL I METODE

4.1. Eksperimentalne životinje

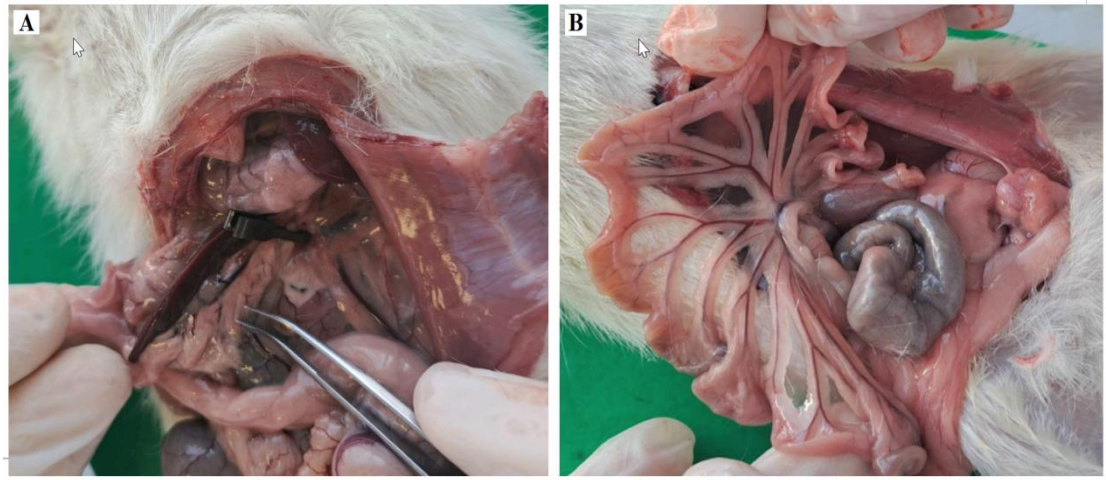
Istraživanje je sprovedeno u Centru za Biomedicinska istraživanja Medicinskog fakulteta, Univerziteta u Banjoj Luci, tokom juna i septembra 2023 godine. Pre početka izvođenja istraživanja, dobijena je saglasnost Etičkog odbora za rad sa eksperimentalnim životinjama i dobrobiti životinja Medicinskog fakulteta Univerziteta u Banjoj Luci, koji ima nadležnost za periodičan nadzor. Broj rešenja Etičkog odbora je: 18/1. 331-3/23 od 06.06.2023 godine. Sav tretman životinja je sproveden u skladu sa zakonom o zaštiti i dobrobiti životinja RS ("Službeni glasnik Republike Srpske" br. 111/08), Direktivom Evropskog parlameta (2010/63/EU) za izvođenje eksperimenata na životinjama, i smernicama ARRIVE (*Animal Research:Reporting of In vivo experiments*). Muške jedinke *Wistar albino* soja pacova težine 280-320 grama, starosti 8-12 nedelja, su odgajane u kontrolisanim laboratorijskim uslovima na sobnoj temperaturi od 21 ± 2°C, pri vlažnosti vazduha 55 ± 5%, i 12 h ciklusu svetla i tame uz slobodan pristup hrani i vodi- *ad libitum.*

4.2. Eksperimentalne grupe

Ukupno su 24 životinje ravnomerno raspoređene u 4 jednake grupe. Sve životinje su podvrgnute medijalnoj laparotomiji u uslovima opšte anestezije koja je postignuta intraperitonealnom aplikacijom kombinacije ketamina (90 mg/kg) i ksilazina (10 mg/kg). Šem (sham) grupa je korišćena kao kontrolna (Kontrolna grupa, N=6). Ishemija/ reperfuzija grupa je korišćena kao model bolesti AMI sa klemovanjem gornje mezenterične arterije (SMA) tokom 30 minuta uz 90 minuta reperfuzije (I/R grupa, N = 6). LS grupa je primila levosimendan u dozi 1mg/kg (intraperitonealno, i.p.), i podvrgnuta je istoj šem proceduri (LS grupa, N=6). LS+I/R grupa je primila levosimendan 30 minuta pre izazivanja I/R povrede (LS+I/R grupa, N=6). Dizajn eksperimentalne studije je prikazan na slici 8, dok je način klemovanja kao i izgled mezenterijalne arterije nakon okluzije prikazan na slici 9. Kontrolna i I/R grupa su primile dimetilsulfoksid (DMSO) kao rastvarač za levosimendan. Na kraju eksperimenta sve životinje su žrtvovane iskrvarenjem, a uzorci krvi, terminalnog ileuma, srca, pluća i bubrega su sačuvani za dalje biohemijske, patohistološke, morfometrijske, imuno-histohemijske i molekularno-genetičke analize



Slika 8. Ilustracija dizajna eksperimentalne studije. Šema pokazuje različite eksperimentalne grupe i vreme intervencije, aplikovanje supstanci, medijalnu laparotomiju, ishemiju i reperfuziju. Slika je kreirana pomoću BioRender-a.



Slika 9. Reprezentativne fotografije pokazuju: **A**). SMA je prikazana pažljivom preparacijom od okolnog tkiva. Izolovana SMA je okludirana atraumatskim arterijskim mikroklampom (buldog) na njenom račvanju iz aorte. **B**) Ishemija tankog creva je verifikovana tamnom prebojenošću crevnih vijuga (pretežno terminalnog ileuma), gubitkom pulsacija i vidljivom atonijom creva (posle 30 minuta okluzije SMA). Peritoneum je zaštićen korišćenjem gaza natopljenih toplim fiziološkim rastvorom kako bi se smanjila evaporizacija i gubitak tečnosti.

4.3. Uzimanje uzoraka krvi i njihova analiza

Uzorci krvi su uzimani iz aorte na kraju reperfuzije, u epruvete za serum i epruvete za plazmu sa antikoagulansom. Uzorci seruma su ostavljani da se napravi koagulum, a potom su centrifugirani 5 minuta na 3000 obrtaja. Lizat eritrocita je dobijen posle odvajanja plazme, pri čemu su eritrociti ispirani tri puta u hladnom fiziološkom rastvoru, te su nakon pripreme uzorci sačuvani na -80 ^{0C} do analize.

Markeri oksidativnog stresa su mereni u plazmi, eritrocitnom lizatu i u homogenatu terminalnog ileuma. Prooksidativni markeri u plazmi, uključujući H_2O_2, NO_2^-, O_2^- su mereni metodom Pick i Keisari [227], Green metodom i Nitro Blue Tetrazolium (NBT) redukcionom metodom [228,229]. Lipidna peroksidacija je evaluirana merenjem TBARS korišćenjem 1% TBA i 0.05 M sodium natrijum hidroksida (NaOH), sa očitavanjima snimljenim na 530 nm [230]. Nivo antioksidanata u eitrocitnom lizatu uključujući CAT, SOD i GSH je meren spektrofotometrijski prema Beutlerovoj Metodi [231-233].

4.4.Uzimanje uzoraka BALF-a i analiza

Nakon uzimanja krvi, ispreparisana je traheja u koju je postavljena mala kanila i bronho-alveolarni lavaž *(Bronchoalveolar Lavage Fluid*, BALF) je sakupljen instilacijom i aspiracijom 0,7 ml hladnog fosfatnog puferisanog rastvora tri puta. Uzorci su centrifugirani na 5000 obrtaja, na 4^0C , tokom 10 minuta. Na ovaj način je dobijen supernatant za analizu

markera oksidativnog stresa (TBARS, NO_2^-, CAT, SOD, GSH). Azot dioksid je meren Griess-ovom metodom [234]. Aktivnosti enzima SOD i CAT su merene Madesh i Aebi metodama, a GSH Ellman-ovom metodom [235-237].

4.5. Uzimanje uzoraka tkiva, histopatološka ispitivanja i morfometrijska analiza

Nakon eutanazije ekstrahovani su terminalni ileum, srce, pluća i bubrezi. Posle 48 sati fiksacije u 4% formaldehidu, tkivni uzroci su obrađeni koristeći Leica TP 1020 tkivni procesor i ugrađeni u parafinske blokove. Preseci su izrezani na debljinu od 4 μm pomoću Rotatory3003 pfm mikrotoma i obojeni su rutinskim bojenjem hematoksilinom i eozinom kao i Alcian Blue Stain kitom (pH 2.5, mucin stain, Abcam). Uzorci su potom analizirani pod Leica DM 6000 binoкularmim mikroskopom koji je bio opremljen Leica DFC310FX kamerom. Za morfometrijsku analizu LAS V4.12 softver je korišćen za 10x uvećanje na deset vidnih polja za svaki uzorak. Skor oštećenja tkiva je određivan semikvantitativno prema postojećim publikacijama [238-241]. Numerička gustina polja određena je za preparate tkiva obojene Alcijan blu bojom. Ova gustina odražava broj goblet (peharastih) ćelija u terminalnom ileumu u odnosu na celu površinu tkiva. Vidno polje je prvo bilo izračunato u kvadratnim milimetrima, a potom je numerična gustina polja (NA) bila izračunata kao količnik broja struktura ili ćelija (N) i površine vidnog polja (A), pri čemu je NA = N/A. Dobijene vrednosti su izražene u procentima.

4.6. Imunohistohemijske analize

Aktivacija specifičnih apoptotičkih puteva je evaluirana tako što smo koristili imunohistohemijska bojenja primarnim antitelima za NF-κB, CC3, Nrf2 i HO-1. Inflamatorni markeri su procenjeni imunohistohemijskom analizom koristeći primarna antitela na CD68 kako bi se potvrdilo prisustvo infiltrata imunoloških ćelija i kontinuirani imunološki odgovor, kao i primarna antitela kako bi se identifikovala inflamacija posredovana citokinima. Antitela su nanesena na uzorke i inkubirana tokom noći na 4°C u vlažnoj komori. Detekcija je postignuta korišćenjem sekundarnih antitela konjugovanih sa peroksidazom rena (HRP) i polivalentnog sistema za detekciju HRP (*UltraVision Detection System HRP Polymer & DAB Plus Chromogen, Thermo Fisher Scientific, USA*). Mesta vezivanja antitela postala su vidljiva nakon bojenja hromogenom 3,3′-diaminobenzidine tetrahydrochloride (DAB), i uzorci su potom obojeni hematoksilin- eozin metodom.

Apoptoza je takođe analizirana TUNEL metodom koristeći komercijalni kit za detekciju (*TUNEL In Situ Kit, Elabscience, China; catalogue number: E-CK-A331*) prema uputstvima proizvođača. Ukratko, parafinski preparati su deparafinizirani, rehidrirani i tretirani sa proteinazom K za permeabilizaciju. Posle ispiranja tkivo je inkubirano reaktivnim rastvorom koji sadrži TdT enzim i obeležene nukleotide na 37°C tokom 60 min. Potom je dodat Streptavidin-HRP konjugat a signal je viđen korišćenjem DAB substrata pri čemu su apoptotske ćelije viđene kao smeđe obojena jedra. Preseci su kontrastno obojeni hematoksilinom, dehidrirani i montirani za analizu svetlosnom mikroskopijom. Apoptotski indeks (AI) koji predstavlja procenat apoptotskih ćelija izračunat je prema sledećoj formuli:
AI (%) = (Broj TUNEL-pozitivnih ćelija× 100) / Ukupan broj ćelija.

Svi uzorci su ispitivani pomoću Leica DM2500 optičkog mikroskopa i snimljeni MC170HD kamerom pri uvećanju od 400X. Mikrofotografije su arhivirane u TIFF formatu. Imunološki odgovor je analiziran pomoću Fiji softvera (*National Institutes of Health, Bethesda, MD, USA*), s fokusom na broj DAB pozitivnih ćelija i srednju optičku gustinu u posmatranom tkivu creva. Rezultati su prikazani kao srednja optička gustina ± standardna devijacija za 10 vidnih polja po pacovu i srednja optička gustina je upoređena između grupa.

4.7.Molekularno-genetičke analize

U okviru istraživanja urađeni su i molekularno-genetički testovi koji obuhvataju analizu relativne genske ekspresije ukupne genomske RNK izolovane iz ileuma pacova Wistar soja. Praćena je genska ekspresija SOD kao i CAT gena u 4 eksperimentalne grupe. Prva grupa obuhvatala je kontrolni uzorak 3 pacova. Drugu eksperimentalnu grupu činili su pacovi koji su podvrgnuti I/R tretmanu. Treća grupa pacova je tretirana samo levosimendanom, dok je poslednja grupa tretirana levosimendanom i I/R zahvatom.

4.7.1. Izolacija ukupne genomske RNK

Nakon žrtvovanja pacova i uzimanja dela ileuma, te prečišćavanja fiziološkim rastvorom sa fosfatnim puferom (PBS), isečak tkiva je stavljen u rastvor za sprečavanje degradacije RNK molekula u odnosu 1:5. Potom je rađena izolacija ukupne genomske RNK pomoću PureLink RNA mini kit, prema upustvima proizvođača, a koja obuhvataju sledeće korake:

1. Priprema pufera za homogenizaciju tkiva koji podrazumeva dodavanje pufera za lizu i 2-merkaptoetanola. Po svakom uzorku od 30 mg tkiva dodavno je 6 µL 2-merkaptoetanola i 600 µL pufera za lizu.
2. Nakon pripreme pufera za homogenizaciju, tkivo je homogenizovano na ledu pomoću Rotor-Stator homogenizatora na 10000xg u trajanju od 30-40 sekundi. Potom, homogenat tkiva je centrifugiran na sobnoj temperaturi u trajanju od 5 minuta na 2600xg.
3. Supernatant se odvoji u čistu sterilnu tubu, te se dodaje 70% alkohol u zapremini jednakoj zapremini supernatanta.
4. Nakon prenošenja u čiste kertridž tube i centrifugiranja na 14000xg u trajanju od 15 sekundi, dodaje se 600 µL pufera 1 za ispiranje proteina, lipida, ostataka membrana i molekula DNK.
5. Zatim sledi ispiranje puferom 2 u zapremini od 500 µL čiji je cilj dodatno uklanjanje soli, deterdženata, te malih organskih molekula. Potom je nephodno centrifugirati 14000xg u trajanju od 15 sekundi.
6. Kao krajnji korak dodaje se 50 µL sterilne vode, te se centrifugira na 15000xg 120 sekundi na sobnoj temperaturi. Uzorak je spreman za čuvanje na -20ºC ili kod dužeg čuvanja na -80ºC.

4.7.2.Kvantifikacija izolovane ukupne genomske RNK

Kvantifikacija izolovane RNK rađena je pomoću Qubit4 fluorometra upotrebom qubit kita za kvantifikaciju sledećim redosledom:

1. Za pravljenje standarda 1 i 2 koji predstavljaju poznatu koncentraciju ukupne humane genomske RNK, dodavano je 190 µL pufera i 10 µL reagensa, za svaki standard.

2. Za svaki uzorak koji je kvantifikovan, radna solucija je pravljena tako što je dodavano 199 µL pufera i 1 µL reagensa, te je od radne solucije od 200 µL, u svaki uzorak pojedinačno dodavano 199 µL radne solucije i1 µL RNK.

4.7.3. Reverzna transkripcija

Nakon što je izolovana i kvantifikovana iRNK, neophodno je napraviti razblaženja tako da koncentracija RNK iznosti 20 ng/ µL. Reverzna transkripcija predstavlja prevođenje jednolančane RNK u komplementarnu jednolančanu DNK. Reverzna transkripcija je rađena na klasičnom PCR aparatu tako da su uslovi reakcije bili: korak 1: 25ºC 10 minuta, korak 2:

37ºC 120 minuta i korak 3: 85ºC 5 minuta. Za svaki uzorak dodavane su sledeće komponente High Capacity DNA reverznog kita sa inhibitorom Rnase:

1. 10X RT pufer u zapremini od 2 μL
2. 25X dNTP miks (100 mM) 0,8 μL
3. 10x RT nasumični prajmeri 2 μL
4. MultiScribe reverzna traskriptaza 1 μL
5. Inhibitor Rnase 1 μL
6. Voda 3,2 μL
7. 10 μL RNK uzorka

4.7.4. Lančana reakcija polimeraze u realnom vremenu/ kvantifikacija relativne genske ekspresije metodom ΔΔCt

RT PCR ili kvantitativni PCR (qPCR**)** predstavlja metodu kojom se istovremeno amplifikuje DNK i kvantifikuje njena količina u realnom vremenu**.** Za razliku od klasične PCR, kod qPCR se nakon svakog ciklusa merenja fluorescence prati rast DNK, što omogućava određivanje početne količine materijala. Za analizu ekspresije gena cDNA se koristi kao šablon u qPCR. U relativnoj kvantifikaciji koristi se ΔCt (razlika između Ct ciljnog i referentnog gena) i ΔΔCt (razlika ΔCt tretirane i kontrolne grupe), dok se relativna ekspresija računa formulom 2^-ΔΔCt**.** Real-time PCR funkcioniše tako što se u toku amplifikacije ciljane DNK koristi specifična TaqMan proba**,** koja je na 5' kraju označena fluorescentnim reporterom, a na 3' kraju ima prigušivač koji blokira fluorescenciju. Kada se prajmeri vežu za ciljnu sekvencu i Taq polimeraza počne sintezu novog lanca, njena 5'-3' egzonukleazna aktivnost uklanja probu, što dovodi do odvajanja reportera od prigušivača. Odvajanje omogućava emitovanje fluorescencije, koja se detektuje od strane aparata. Kako se reakcija odvija ciklus po ciklus, intenzitet fluorescencije raste proporcionalno količini amplifikovanog proizvoda, što omogućava praćenje i kvantifikaciju početne količine cDNA. U ovom istraživanju analizirana je relativna genska ekspresija SOD1 rn00566938 i CAT1 rn00560930_m1. Za svaki analizirani uzorak pripremljena je smeša koja se sastoji od 10 μL master miksa, 1 μL TaqMan eseja za gensku ekpresiju, 7 μL vode i 2 μL cDNK. Za referentni gen korišćen je GAPDH. Uslovi PCR reakcije bili su: inkubacija 50 ºC 2 minuta; enzimska aktivacija na 95 ºC 20 sekundi, denaturacija 95 ºC 3 sekunde, vezivanje prajmera 60 ºC 30 sekundi. Poslednja 2 koraka ponovljena su u 40 ciklusa.

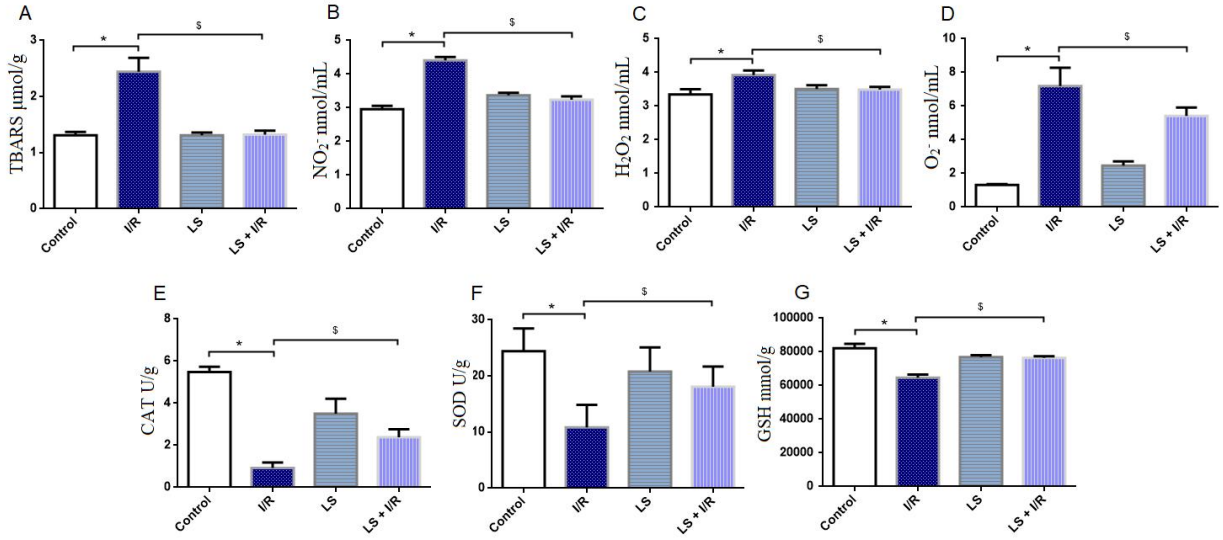
4.8. Statistička obrada podataka

Statistička analiza je izvedena pomoću IBM-SPSS Statistic verzije 20.0 softvera (SPSS,Inc., Chicago Il, USA), dok je GraphPad Prism 6.0 softver korišćen za grafičke prezentacije. ANOVA test je korišćen za poređenja parametrijskih karakteristika a Kraskal-Volis test za poređenje neparametrijskih karakteristika između grupa. Tukijev i Bonferonijev test su poslužili za post-hok analize. Rezultati su prikazani kao srednja vrednost i standardna greška. Nivo značajnosti je bio $p < 0.05$. Dobijeni rezultati su prikazani grafički.

5. REZULTATI

5.1. Efekti primene levosimendana na markere oksidativnog stresa u plazmi i lizatu eritrocita tokom AMI

Značajan porast u lipidnom peroksidacionom indeksu (TBARS) je viđen u I/R grupi u poređenju sa kontrolnom grupom. Ovaj primetni porast u TBARS-a u plazmi je povezan sa povišenim nivoima svih testiranih prooksidativnih markera, H_2O_2, O_2^-, and NO_2 ($p < 0.05$). I/R grupa takođe pokazuje smanjenje nivoa antioksidativnih enzima katalaze (CAT), superoksid dismutaze (SOD) i redukovanog glutationa (GSH), mereno u lizatu eritrocita. Međutim, pretretman levosimendanom ublažio je efekte mezenterične I/R lezije što je dokazano značajnim smanjenjem nivoa TBARS-a i prooksidativnih enzima u plazmi ($p<0.05$). Pored toga, levosimendan je pokazao antioksidativni učinak povećanjem nivoa antioksidativnih enzima: SOD, CAT i GSH u poređenju sa I/R grupom (Slika 10).



Slika 10. Efekti levosimendana na markere oksidativnog stresa u plazmi i eritrocitnom lizatu pacova. (A) TBARS (µmol/g); (B) NO_2^- (nmol/ml); (C) H_2O_2 (nmol/ml); (D) O_2^- (nmol/ml); (E) CAT (U/g); (F) SOD (U/g); (G) GSH (mmol/g). Mezenterična I/R lezija dovodi do značajnog porasta indeksa lipidne peroksidacije (TBARS) i prooksidativnih markera: vodonik peroksida (H_2O_2), nitrita (NO_2^-) i superoksid anjon radikala (O_2^-), uz smanjenje antioksidativnih enzima (CAT, SOD i GSH), dok pretretman levosimendanom značajno smanjuje nivo TBARS i prooksidativnih markera u plazmi i povećava nivo antioksidativnih enzima u poređenju sa I/R grupom. Podaci su izraženi kao srednja vrednost ± SD. *$p < 0.05$ upoređujući kontrolnu (C) grupu sa I/R grupom; $^{\$}p < 0.05$ upoređujući I/R sa LS + I/R grupom.

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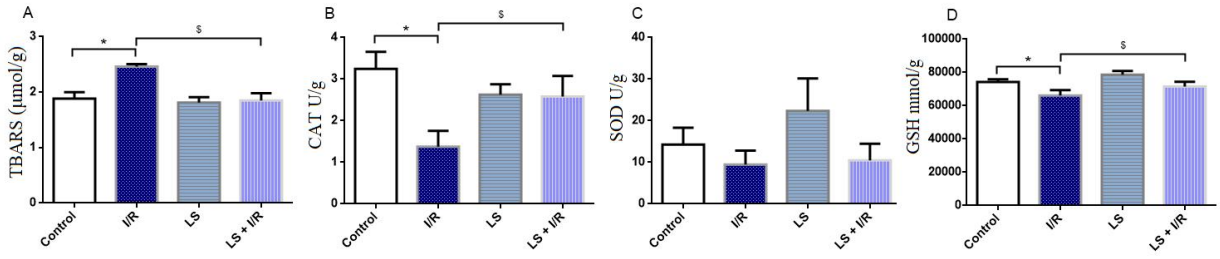
5.2. Efekti primene levosimendana na markere oksidativnog stresa u homogenatu terminalnog ileuma tokom AMI

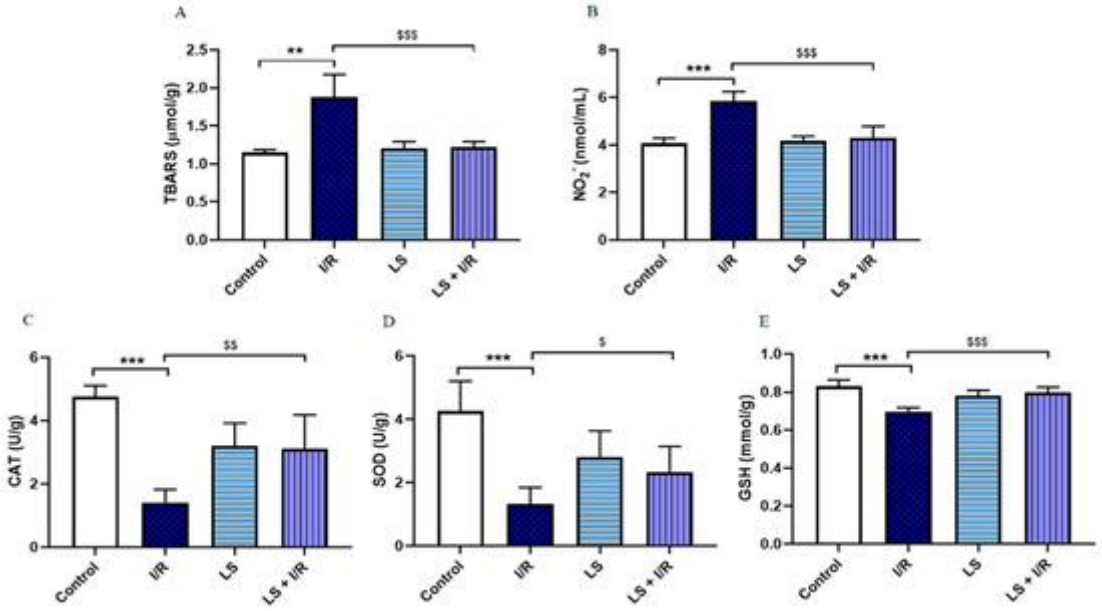
Dosledno sa nalazima u plazmi, nivo TBARS je bio značajno povišen u homogenatu terminalnog ileuma pacova u I/R grupi u poređenju sa kontrolnom grupom. Pored toga CAT, SOD i GSH aktivnost je bila značajno smanjena u homogenatu terminalnog ileuma pacova podrgnutih I/R leziji u poređenju sa kontrolnom grupom ($p < 0.05$). Povišene vrednosti TBARS-a su normalizovane i antioksidativne aktivnosti CAT i GSH su značajno poboljšane u I/R grupi koja je prethodno tretirana levosimendanom (LS+I/R) ($p < 0.05$). Međutim, pretretman levosimendanom nije demostrirao blagotvorni efekat na aktivnost SOD u homogenatu terminalnog ileuma (slika 11).



Slika 11. Efekti levosimendana na markere oksidativnog stresa u homogenatu terminalnog ileuma pacova. TBARS (μmol/g); (B) CAT (U/g); (C) SOD (U/g); (D) GSH (mmol/g). TBARS nivoi su značajno povišeni a nivoi CAT, SOD, i GSH aktivnosti su značajno smanjeni u I/R grupi u poređenju sa kontrolnom grupom, dok pretretman levosimendanom značajno smanjuje nivo TBARS-a i poboljšava nivo CAT i GSH aktivnosti, ali nema takav efekat na aktivnost SOD. Podaci su izraženi kao srednja vrednost ± SD. *$p < 0.05$ poređenje kontrolne grupe sa I/R grupom; $^{\$}p < 0.05$ poređenje I/R grupe sa LS + I/R grupom.

5.3. Efekti primene levosimendana na markere oksidativnog stresa u BALF-u.

Levosimendan je uticao na markere oksidativnog stresa u ispirku bronhoalveolarne lavaže kod pacova. U I/R grupi, TBARS ($p<0.01$) i $N0_2^-$ ($p< 0,001$) bili su značajno povišeni, dok su aktivnosti SOD, CAT i GSH bile značajno smanjene u poređenju sa kontrolnom grupom ($p<0.001$). Pretretman levosimendanom ublažio je porast TBARS-a i NO_2^- ($p<0.001$) i značajno vratio aktivnosti CAT ($p<0.01$), SOD ($p<0.05$) i GSH ($p<0.001$) prema kontrolnim nivoima (Slika 12).

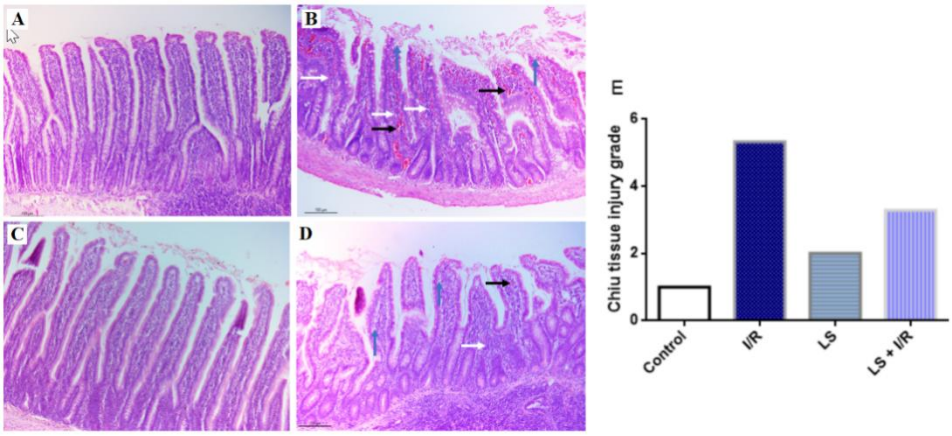


Slika 12. Efekti levosimendana na markere oksidativnog stresa u BALF-u pacova. (**A**) TBARS (µmol/g); (**B**) NO_2^- (nmol/mL); (**C**) CAT (U/g); (**D**) SOD (U/g); (**E**) GSH (mmol/g). TBARS nivoi su značajno povećani i aktivnosti CAT, SOD i GSH su značajno smanjene u I/R grupi u komparaciji sa kontrolnom grupom, dok pretretman levosimendanom značajno smanjuje TBARS nivoe i povećava CAT, SOD i GSH aktivnosti. Podaci su izraženi kao srednja vrednost ± SD. Control(kontrolna grupa) (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6). ** $p < 0.01$; *** $p < 0.001$ upoređujući kontrolnu sa I/Rgrupom; $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$, upoređujući I/R sa LS+I/R grupom.

Levosimendan smanjuje efekte mezenterične I/R lezije na oksidativni stres u krvi, intestinalnom tkivu i u BALF-u.

5.4. Efekti levosimendana na epitelne ćelije terminalnog ileuma pacova koje su izložene I/R leziji

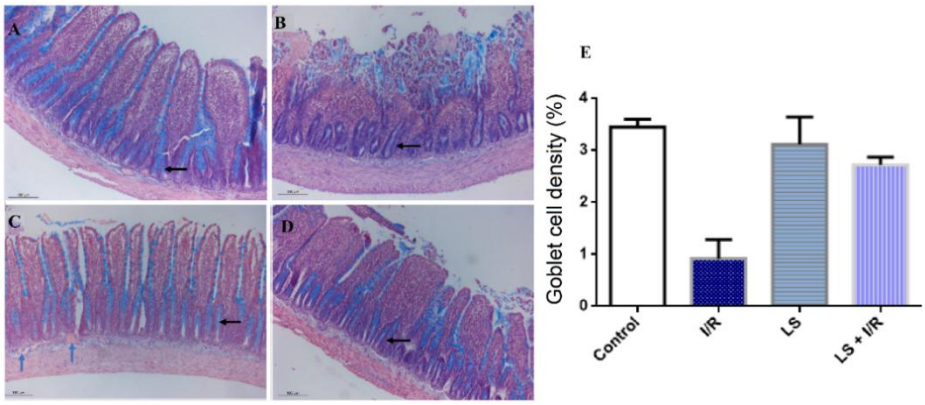
Kao što je pokazano na slici 13, preseci ileuma u I/R grupi prikazuju poremećaj- disrupciju arhitekture crevnih resica (*villi intestinales*) što je povezano sa značajnom podizanjem epitela, zapaljenskom infiltracijom i krvarenjem. LS grupa pokazuje histološke nalaze slične kontrolnoj grupi. Pretretman levosimendanom pokazuje značajno smanjenje dezintegracije crevnih resica uz smanjenje zapaljenske infiltracije i krvarenja u lamini propriji u poređenju sa I/R grupom. Stepen lezija terminalnog ileuma, baziran na Chiu skoru je korišćen kao semikvantitativna analiza za sagledavanje razlika između grupa obojenih hematoksilinom i eozinom (Slika 13).



Slika 13. Reprezentativne mikrofotografije preseka terminalnog ileuma pacova obojenih hematoksilinom i eozinom (uvećanje 10×, skala bar = 100 μm). (A) Očuvana arhitektura crevnih resica terminalnog ileuma u kontrolnoj grupi. (B) I/R grupa pokazuje ozbiljno narušavanje arhitekture crevnih resica sa ekstenzivnim podizanjem epitela (plava strelica), zapaljenskom infiltracijom (bela strelica) i krvarenjem (crna strelica) kroz laminu propriju. (C) Levosimendan (LS) grupa pokazuje normalnu histološku sliku terminalnog ileuma. (D) LS + I/R grupa pokazuje znatno manje oštećenje sluznice creva sa blažom inflamatornom infitracijom i krvarenjem u poređenju sa I/R grupom. (E) Lezija terminalnog ileuma je procenjena prema Chiu skoru.

Levosimendan značajnu smanjuje leziju creva uz blažu inflamatornu infiltraciju kao i pojavom manjeg krvarenja.

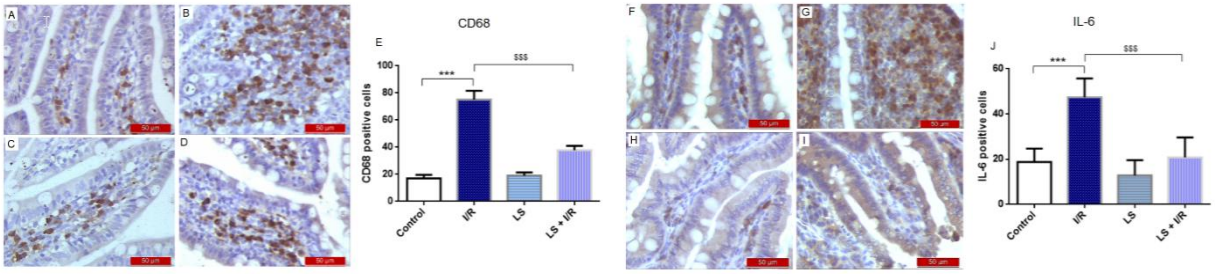
Histopatološka ispitivanja preparata terminalnog ileuma pacova obojenih sa Alcijanskim plavim bojenjem (*Alcijan blue)* otkrivaju nalaze slične onima koji su prethodno viđeni prilikom hematoksilin i eozin bojenja. Kontrolna grupa pokazuje normalnu histologiju terminalnog ileuma sa tipičnom distribucijom peharastih (goblet) ćelija u Liberkinovim kriptama i u epitelnom sloju sluznice tankog creva. U I/R grupi se uočava destrukcija crevnih resica, zapaljenski infiltrati i odsustvo goblet ćelija u kriptama. U LS grupi je viđena normalna distribucija goblet ćelija, dok je pretretman levosimendanom u I/R grupi (LS+ I/R) izazvao većinom normalnu distribuciju goblet ćelija, sa mestimičnim odsustvom u pojedinim kriptama (Slika 14).



Slika 14. Efekti levosimendana na histopatološku sliku intestinalnog tkiva u mezenteričnoj I/R leziji. Poprečni preseci tankog creva, uvećanje x100 (Alciansko plavo bojenje, skala bar 100 μm).(A) Kontrolna grupa pokazuje normalnu histologiju terminalnog ileuma, sa tipičnom distribucijom goblet ćelija u Liberkinovim kriptama(crna strelica) i u epitelnom sloju sluznice. (B) I/R grupa pokazuje destrukciju apikalnih delova crevnih resica, zapaljenski infiltrat pri bazi i odsustvo goblet ćelija u kriptama (crna strelica). (C) LS grupa pokazuje zadebljanu submukozu sa dilatiranim krvnim sudovima (plava strelica) i histološki normalnu sluznicu sa goblet ćelijama u kriptama (crna strelica). (D) LS + I/R grupa pokazuje dilatirane crevne resice, većinom sa normalnom distribucijom goblet ćelija i sa mestimičnim odsustvom u pojedinačnim kriptama (crna strelica). (E) Numerička površinska gustina goblet ćelija (%), pokazuje značajnu prezervaciju u LS+I/R grupi.

4.5. Efekti primene levosimendana na inflamatorni odgovor tokom AMI

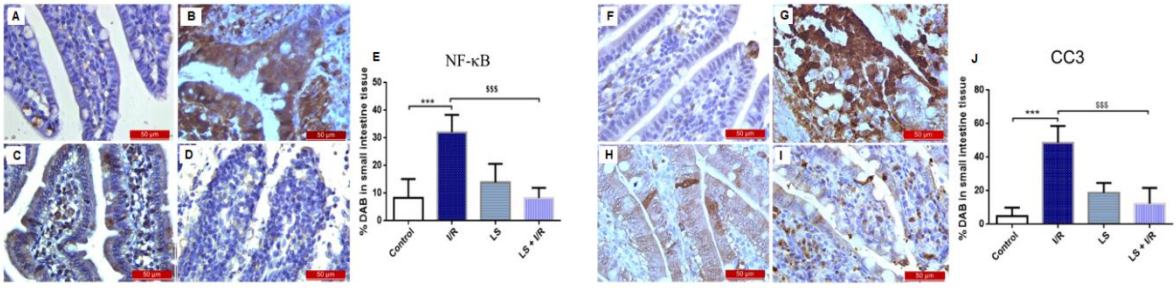
Pored izazivanja strukturnog oštećenja i smanjenja antioksidativnih odbrambenih mehanizama, intestinalna I/R lezija dovodi i do izraženog povišenja inflamatornih medijatora. Imunoreaktivnost CD68 i IL-6 ukazuje na njihovu ključnu ulogu u pokretanju i održavanju zapaljenja tokom I/R lezije. U kontrolnoj grupi samo mali broj CD68 pozitivnih makrofaga i IL-6 pozitivnih ćelija je viđen izvan krvnih sudova, u stromi crevnih resica. Suprotno tome I/R grupa pokazuje značajno povećanje CD68 pozitivnih makrofaga i IL-6 pozitivnih ćelija, pretežno lokalizovanih u erozijama pri vrhovima oštećenih crevnih resica. Levosimendan grupa pokazuje imunoreaktivnost uporedivu sa kontrolnom grupom, dok pretretman levosimendanom značajno redukuje infiltraciju makrofaga i IL-6 ekspresiju u I/R lediranom tkivu terminalnog ileuma (Slika 15).



Slika 15. Ekspresije CD68 (levi panel) i IL-6 (desni panel) u tkivu terminalnog ileuma pacova. Podaci su izraženi kao srednja vrednost ± SD. Reprezentativne imuhohistohemijske slike sa 400× uvećanjem. (A, F) Kontrolne grupe. (B, G) Značajno povećanje broja CD68- pozitivnih makrofaga (B) i IL-6- pozitivnih ćelija (G) je uočeno u I/R grupama. (C, H) Levosimendan (LS) grupe pokazuju imunoreaktivnost sličnu kontrolnim grupama. (D, I) LS + I/R grupe pokazuju značajno smanjenje imunoreaktivnosti i za makrofage i za IL-6 . (E, J) procenat DAB- obojenih ćelija u tankom crevu ukazuje na proporciju CD68 pozitivnih (E) i IL-6 pozitivnih (J) ćelija . ***$p < 0.001$ upoređujući kontrolnu sa I/R grupom; $^{\$\$\$}p < 0.001$ upoređujući I/R sa LS + I/R grupom.

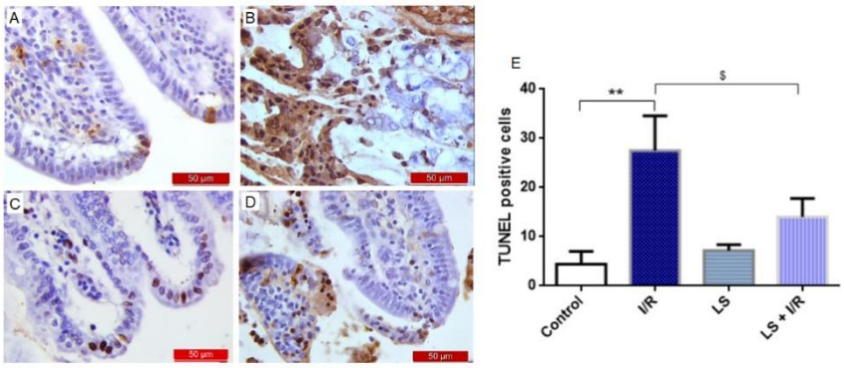
4.6. Efekti primene levosimendana na apoptozu epitelnih ćelija ileuma tokom AMI

Imunohistohemijska analiza je otkrila značajno povećanje ekspresije Nuklearnog faktora kapa Beta (NF-κB) i cepane kaspaze 3 (CC3) u I/R grupi. Povećanje ekspresije NF-kB i CC3 u I/R grupi, ukazuje da ovi markeri igraju ključnu ulogu u patofiziološkom odgovoru na leziju ileuma pacova i apoptozu. Pretretman levosimendanom (1mg/kg) značajno smanjuje crevnu epitelijalnu ćelijsku apoptozu indukovanu I/R lezijom, što je dokazano smanjenjem ekspresije NF-kB i CC3. Rezultati pokazuju da pretretman levosimendanom može ublažiti oštećenja nastala usled mezenterične I/R lezije. (LS + I/R vs. I/R, $p< 0.001$) (Slika 16). Levosimendan smanjuje apoptozu crevnih epitelnih ćelija indukovanu I/R lezijom.



Slika 16. Imunohistohemijski rezultat ekspresije NF-kB (levi panel) i CC3 (desni panel), u tankom crevu pacova. Podaci su izraženi kao srednja vrednost ± SD. Reprezentativne imunohistohemijske slike sa 400× uvećanjem. (A, F) Kontrolne grupe. (B, G) Intenzivno citoplazmatsko bojenje NF-κB (B) i CC3 (G) u crevnim epitelijalnim ćelijama u I/R grupama ukazuje na aktivnost povezanu sa apoptozom. (C, H) Levosimendan (LS) grupe ne pokazuju značajnu razliku u imunoreaktivnosti na markere apoptoze u poređenju sa kontrolnim grupama. (D, I) Izraženo je smanjenje apoptoze u epitelnim ćelijama i crevno oštećenje u LS + I/R grupama. (E, J) Procenat DAB obojenih ćelija u tkivu tankog creva ukazuje na proporciju NF-κB-pozitivnih (E) i CC3-pozitivnih (J) celija. ***$p < 0.001$ upoređujući kontrolnu sa I/R grupom; $^{\$\$\$}p < 0.001$ upoređujući I/R sa LS + I/R grupom..

Kako bi se dodatno potvrdili antiapoptotski efekti levosimendana, sprovedeno je TUNEL bojenje. Levosimendan je smanjio apoptotski indeks što je primećeno kao značajno smanjen broj uglavnom TUNEL pozitivnih epitelnih ćelija tkiva tankog creva (Slika 17).

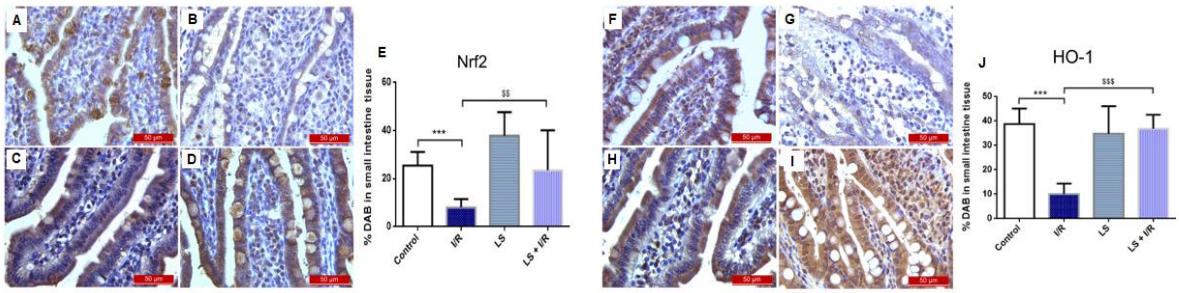


Slika 17. Levosimendan (LS) inhibira apoptozu u crevnom tkivu pacova tokom I/R mezenterične lezije, detektovano TUNEL bojenjem (A) Kontrolna grupa. (B) Značajan porast u DNK fragmentaciji je uočen u I/R grupi, što je dokazano intenzivnim braon TUNEL bojenjem. (C) LS grupa je pokazala sličnu pojavu i distribuciju TUNEL pozitivnih ćelija kao i u kontrolnoj grupi. (D) Pretretman levosimendanom je prevenirao I/R indukovanu nuklearnu apoptozu. (E) Kvantitativna analiza apoptotskih ćelija je izvedena na imunohistohemijski obojenim presecima crevnog tkiva pacova. **$p < 0.01$ upoređujući kontrolnu sa I/R grupom; $^{\$}p < 0.05$ upoređujući I/R sa LS + I/R grupom.

Levosimendan smanjuje broj TUNEL pozitivnih ćelija.

5.7. Efekti levosimendana na regulaciju Nrf2 i HO-1 signalnog puta tokom AMI

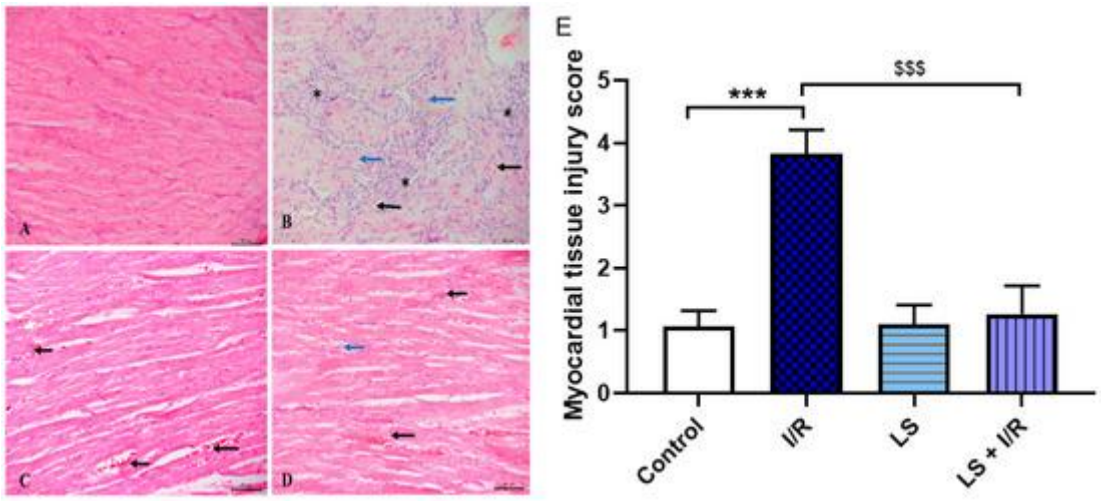
Rezultati ukazuju da prekomerna ekspresija Nrf2 a takođe i antioksidativni odgovor HO-1 u terminalnom ileumu smanjuju intestinalnu I/R leziju. Kontrolna i grupa sa pretretmanom levosimendanom pokazuju sličnu pojavu i distribuciju Nrf2 i HO-1 pozitivnih ćelija, bez zapaženih značajnih razlika, dok I/R grupa pokazuje značajno smanjenje u imunoreaktivnosti. Levosimendan uspešno podiže nivoe ovih antioksidativnih markera, što je potvrđeno značajnim razlikama između LS+I/R i I/R grupa (Nrf2, $p < 0.01$; HO-1, $p < 0.001$) (Slika 18). Pretretman levosimendanom izaziva bolju regulaciju Nrf2, i HO-1, Nrf2/HO-1 signalnog puta.



Slika 18. Imunohistohemijski rezultati ekspresije Nrf2 (levi panel) i HO-1 (desni panel), tkiva tankog creva pacova. Podaci su izraženi kao srednja vrednost ± SD. Reprezentativne imunohistohemijske slike sa 400× uvećanjem. (A, F) Kontrolne grupe. (B, G) Značajno smanjenje u imunoreaktivnosti za antioksidativne markere Nrf2 i HO-1 je uočeno u intestinalnim epitelnim ćelijama I/R grupa. (C, H) Imunoreaktivnost za oba markera u LS grupama bila je uporediva sa observiranim vrednostima u kontrolnim grupama, bez detektovane značajne razlike. (D,I) Pretretman levosimendanom je značajno povećao nivoe Nrf2 i HO-1 kada se porede I/R grupe. (E, J) Procenat DAB-obojenih ćelija u tankom crevu pokazuje proporciju Nrf2 pozitivnih (E) i HO-1 pozitivnih (J) ćelija. ***$p < 0.001$ upoređujući kontrolnu sa I/R grupom; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ upoređujući I/R sa LS + I/R grupom.

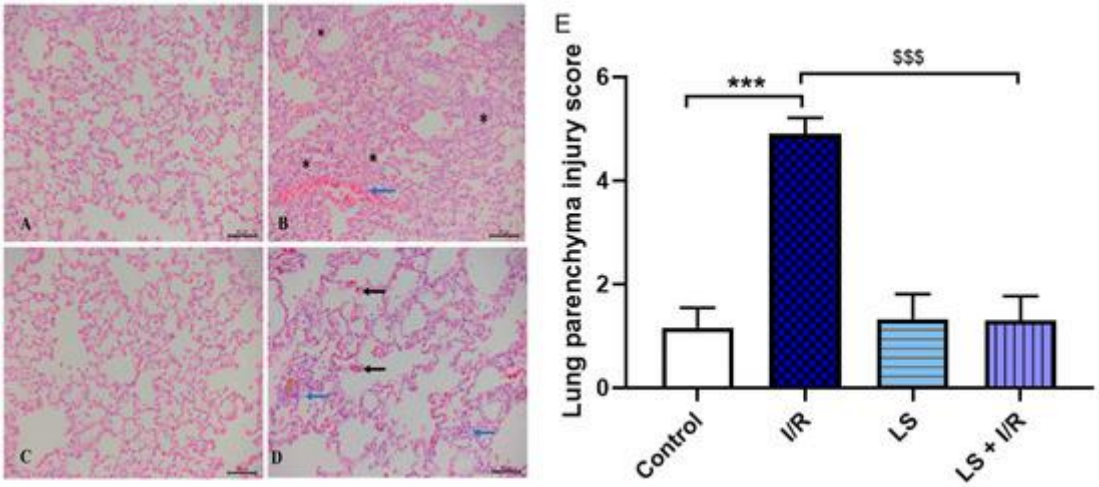
5.8. Efekti primene levosimendana na histopatološke promene u srcu, plućima i bubregu tokom AMI.

Intestinalna I/R lezija izaziva štetu ne samo na crevnom zidu, već i na udaljenim organima kao što su srce, pluća i bubrezi. Lezija miokarda je naizraženija na septumu i u predelu vrha srca. Histopatološka ispitivanja ukazuju na kardiomiocitnu hipertrofiju, ekstenzivne inflamatorne infiltrate, koji se najviše sastoje od limfocita i posledično dolazi do nekroze miokarda. Ordiniranje levosimendana pokazuje značajna vazodilatatorska svojstva, što se uočava pojavom dilatiranih krvnih sudova ispunjenih eritrocitima unutar endomizijuma. Pretretman levosimendanom pruža izraženu kardioprotekciju što se ogleda u boljim rezultatima kad se posmatra skor oštećenja tkiva, integritet kardiomiocita je očuvan, vaskularna dilatacija u endomizijumu se održava a vidljive su samo retke inflamatorne ćelije (Slika 19).



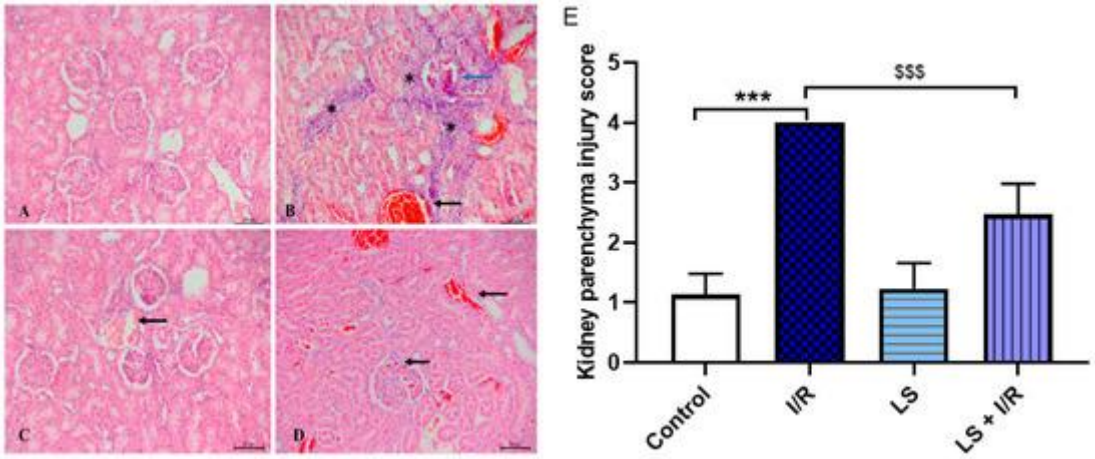
Slika 19. Reprezentativne mikrofotografije srčanog mišića pacova, uzdužni preseci obojeni hematoksilin-eozinom(uvećanje 20 puta, skala bar= 50 μm). (**A**) Očuvana histološka struktura srčanog mišića u kontrolnoj grupi. (**B**) I/R grupa pokazuje gusti inflamatorni infiltrat koji prožima srčani mišić (zvezdica); prisustvo oštećenih kardiomiocita (plava zvezdica) i ektravazaciju eritrocita (crna strelica). (**C**) LS grupa je sačuvala histološku strukturu srčanog mišića sa proširenim krvnim sudovima u endomizijumu (crna strelica). (**D**) LS+I/R grupa je sačuvala histološku strukturu srčanog mišića ; pored dilatiranih krvnih sudova u endomizijumu (crna strelica), prisutne su i rasute inflamatorne ćelije (plava strelica). (**E**) Skor oštećenja srčanog mišića (srednja vrednost ± SD, Control (kontrolna grupa), N = 6; LS, N = 6; I/R, N = 6; LS + I/R, N = 6). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (između naznačenih grupa).

Pluća spadaju u organe koji su najteže pogođeni intestinalnom I/R lezijom. Ovo stanje dovodi do difuznog oštećenja plućnog parenhima koje je praćeno značajnim oštećenjem respiratornog epitela. Skoro sve alveole pokazuju strukturalna oštećenja i infiltrirane su gustim inflamatornim ćelijama. Pored toga, fibrozne promene evidentne su u bazalnim regionima pluća. Pretretman levosimendanom pruža značajnu zaštitu plućnom parenhimu pri čemu se u bazalnim delovima pluća zadržavaju samo retka inflamatorna ćelijska žarišta, dok većina plućnog parenhima ostaje očuvana (slika 20).



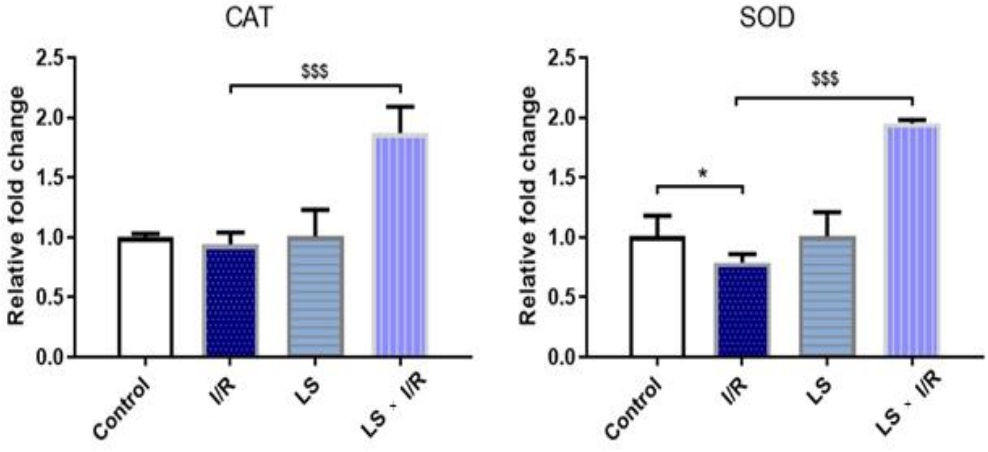
Slika 20. Reprezentativne mikrofotografije plućnog parenhima pacova obojenih hematoksilinom i eozinom(uvećanje 20 puta, skala bar=50 μm). (**A**) Očuvana histološka struktura plućnog parenhima u kontrolnoj grupi. (**B**) I/R grupa pokazuje značajno oštećenje, sa interalveolarnim prostorima ispunjenim inflamatornim infiltratima (zvezdica), uočavaju se dilatirani krvni sudovi i ektravazacija eritrocita (crna strelica).(**C**) LS grupa je očuvala histološku strukturu plućnog parenhima. (**D**) LS + I/R grupa je uglavnom očuvala histološku strukturu plućnog parenhima; prisutne su rasute inflamatorne ćelije (plava strelica) i ekstravazacija eritrocita (crna strelica). (**E**) Skor oštećenja plućnog parenhima (srednja vrednost ± SD, Control (kontrolna grupa) (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6); 10 polja po uzorku). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (između naznačenih grupa).

Bubrežni parenhim, uključujući korteks i medulu takođe je podložan oštećenjima tokom mezenterijalne I/R lezije. Gusti inflamatorni infiltrati prožimaju kortikalni region, uzrokujući potpunu destrukciju glomerula. Iako pretretman levosimendanom pokazuje zaštitni efekat na bubrežni parenhim, analiza oštećenja tkiva otkriva da je njegov uticaj relativno ograničen na ovom tkivu u odnosu na druge organe. Histološki pregled ukazuje na očuvanje glomerula, ali perzistira oštećenje u sabirnim kanalićima unutar medularnog regiona, što je praćeno retkim žarištima inflamatornih ćelija (slika 21).



Slika 21. Reprezentativne mikrofotografije preseka renalnog korteksa pacova obojenih hematoksilinom i eozinom(uvećanje 20 puta, skala bar=50 μm). (**A**) Očuvana je histološka struktura bubrežnog korteksa u kontrolnoj grupi. (**B**) I/R grupa pokazuje gusti inflamatorni infiltrat (zvezdica) u bubrežnom parenhimu; kompletno oštećeni bubrežni korpuskul (plava strelica), dilatirane krvne sudove sa ekstravazacijom eritrocita (crna strelica). (**C**) LS grupa pokazuje očuvanu histološku strukturu bubrežnog korteksa, blago dilatiran krvni sud (crna strelica). (**D**) LS + I/R grupa je očuvala histološku strukturu bubrežnog parenhima uz prisustvo dilatiranih krvnih sudova, uključujući krvne sudove glomerula (crna strelica). (**E**) Skor oštećenja bubrežnog parenhima (srednja vrednost ± SD, Control (kontrolna grupa) (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6); 10 polja po uzorku). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (između naznačenih grupa).

Analiza relativne genske ekspresije CAT i SOD u ileumu pacova pokazala je značajne razlike između tretiranih grupa. Naime, u grupi pacova podvrgnutim ishemija-reperfuzija tretmanu relativna genska ekspresija SOD/CAT je bila snižena u odnosu na kontrolnu grupu, dok je u grupi LS+IR bila značajno povišena u odnosu na kontrolnu grupu. U uzorcima tretiranim levosimendanom relativna genska ekspresija je bila jednaka kontrolnoj grupi.



Slika 22. Efekti levosimendana na relativnu gensku ekspresiju SOD i CAT u akutnoj ishemiji mezenterijuma pacova. Rezultati su predstavljeni kao srednja vrednost ± standardna devijacija, a razlike između grupa su smatrane statistički značajnim pri $p < 0.05$. Zvezdica (*) označava značajne razlike u poređenju sa kontrolnom grupom (*$p < 0.05$), dok simbol ($) označava značajne razlike u poređenju sa I/R grupom ($$$ $p < 0.001$)

6. DISKUSIJA

U ovoj studiji je pokazano da pretretman levosimendanom ima antioksidativne, anti-inflamatorne, antiapoptotske i u celini zaštitne efekte na tanko crevo u eksperimentalnom modelu mezenterične ishemijsko /reperfuzijske lezije kod Wistar pacova.

Sluznica creva je veoma osetljiva na hipoksiju i zahteva veliki priliv kiseonika za održavanje funkcionalnog intergiteta digestivnog trakta. U postprandijalnoj fazi ti zahtevi su značajno veći i iznose više od trećine minutnog volumena. Kada se desi AMI, smanjenje kiseonika inicira intraćelijsku blokadu oksidativne fosforilacije u mitohondrijama sa posledičnim smanjenjem količine ATP-a i inhibicijom ATP zavisnih jonskih pumpi. Ova blokada neizbežno indukuje poremećaj u radu jonskih pumpi i porast intraćelijskog natrijuma i kalcijuma, dovodi do dezintegracije citoskeleta i ćelijskih membrana što konačno dovodi do rupture ćelije i nekroze, posebno ako se ishemija prolongira. Za vreme ishemije ATP se postepeno konvertuje do inozina i hipoksantina koji se akumuliraju u tkivu [242-244].
Nakon što se tkivo reperfuzira, enzim ksantin oksidaza koristi slobodno dostupan kiseonik za oksidaciju hipoksantina. Ovaj proces je povezan sa produkcijom O_2^- koji se potom transformiše dejstvom SOD u H_2O_2, a ovaj molekul se dalje cepa na visoko reaktivni i citotoksični $\cdot OH$. Dodatno, O_2^- može u kombinaciji sa NO da produkuje peroksinitrit ($ONOO^-$). Ova preterana produkcija reaktivnih kiseoničnih i azotnih vrsta (ROS/RNS) započinje fragmentaciju proteina i nukleinskih kiselina, kao i lipidnu peroksidaciju ćelijskih membrana. Svi ovi događaji na kraju dovode do apoptoze i nekroze u nivou epitela [29, 37]. Endogeni antioksidativni sistem (SOD, CAT, GSH) ne može da ukloni veliku količinu slobodnih kiseoničnih radikala nastalih tokom reperfuzijske faze. Mnoge antioksidativne supstance su upravo iz tog razloga istraživane za smanjenje štete uzrokovane intestinalnom I/R lezijom [155, 243, 245-247].

U literaturi su efekti levosimendana tokom I/R lezije ispitivani na tkivu mozga, jetre, srca, pluća i bubrega. S druge strane, mnogo supstanci je ispitivano na modelu I/R lezije na pacovima, model sa privremenom okluzijom SMA pomoću atraumatske kleme. Malo je publikacija koje su ispitivale sveobuhvatne efekte levosimendana tokom mezenterijalne I/R lezije. Većinom su ispitivani parametri oksidativnog stresa i inflamacije. Aygun je ispitivao dejstvo levosimendana na iste parametre i na histopatološke promene creva (kroz analizu

Chiu skora), ali nakon reperfuzije[161]. Polat i saradnici su pored pomenutih parametara ispitivali i nivo NF-kB [226].

Oksidativni stres je u blažem stepenu, što podrazumeva produkciju ROS-a na bazalnom, signalnom nivou, neophodan za mnoge fiziološke procese, kao što je prenos signala između ćelija i adekvatan imunski odgovor, efikasna odbrana od invazivnih mikroba bez razvoja autoimunosti [248]. Ipak, najčešće se radi o oslobađanju većih količina ROS-a u kratkom vremenskom intervalu, što u slučaju mezenterijalne I/R dovodi do apoptoze i nekroze epitelnih ćelija crevnog zida, (narušavanje ćelijske homeostaze), translokacije bakterija i dalje progresije bolesti putem aktivacije makrofaga preko proinflamatornih citokina (IL-1B, IL-6, TNF-alfa) kao i dalje produkcije ROS-a. Poznato je da ROS deluju i kao sekundarni glasnici jer aktiviraju NF-kB i MAPK. Veće količine ROS-a su okidač transkripcije mnogih proinflamatornih medijatora putem aktivacije gena. Ovde se uočava samopojačavajući začarani krug, takozvana pozitivna povratna sprega, gde ROS pojačava inflamaciju koja potom pojačava produkciju ROS-a. NF-κB se sam reaktivira upalnim medijatorima i ROS-om, uzrokujući začarani krug inflamacije i oksidativnog stresa [249-251]. Redoks ravnoteža između ROS-a i antioksidativnih sistema je ključna za održavanje homeostaze. Smanjenje proizvodnje ROS-a i jačanje antioksidativnih mehanizama zaštite su adekvatan način za tretman I/R lezije [252].

NO je u nižim koncentracijama zaštitni faktor preko svojih antioksidativnih, antiinflamatornih i vazodilalatatornih efekata. Uprkos svojim zaštitnim ulogama, NO u visokim koncentracijama indukuje apoptozu epitelnih ćelija crevnog zida tokom intestinalne I/R lezije i time utiče na motilitet creva u smislu pareze koja se viđa u AMI i dovodi do razvoja paralitičkog ileusa u kasnijoj fazi. Visoke koncentracije RNS utiču na prenos signala, produkciju proinflamatornih medijatora, aktiviraju apoptozu, nekrozu i puteve autofagije [37, 253]. U rezultatima koji su postignuti u ovoj studiji, reperfuzioni period bio je praćen značajnim porastom koncentracija prooksidativnih markera (TBARS, H_2O_2, O_2^- i NO_2^-), uz istovremeno smanjenje aktivnosti antioksidativnih enzima SOD, CAT i nivoa neenzimskog antioksidansa GSH. Ovakav biohemijski profil ukazuje na iscrpljivanje antioksidativne zaštite i akumulaciju reaktivnih kiseoničnih i azotnih vrsta, što je karakteristično za reperfuziono oštećenje intestinalnog tkiva [161,226]. Porast TBARS vrednosti odražava povećanu lipidnu peroksidaciju, jedan od glavnih pokazatelja oksidativnog oštećenja ćelijskih membrana [161,195,230]. Povećani nivoi H_2O_2 i NO_2^- dodatno ukazuju na disbalans između produkcije i uklanjanja slobodnih radikala. Smanjenje aktivnosti SOD i CAT enzima, zajedno sa padom

GSH, sugeriše iscrpljivanje endogenog antioksidativnog sistema usled prekomerne potrošnje radi suzbijanja ROS-a, što je u skladu sa literaturnim podacima [37,44,250].

U ovoj studiji levosimendan značajno smanjuje nivo markera oksidativnog stresa (TBARS, H_2O_2, O_2^- i NO_2), i povećava aktivnost antioksidativnih enzima (CAT, SOD) i neenzimskog mehanizma odbrane (GSH), demostrirajući snažan antioksidativni efekat, kako u serumu tako i u homogenatu intestinalnog tkiva. Ovi efekti ukazuju na očuvanje antioksidativnog kapaciteta i smanjenje lipidne peroksidacije, što verovatno proizilazi iz sposobnosti levosimendana da smanji produkciju mitohondrijskih ROS i stabilizuje ćelijske membrane [188,254]. Ovi rezultati su u skladu sa ranijim studijama u kojima je levosimendan pokazao snažna antioksidativna svojstva kao što je u studiji u kojoj je karagenanom indukovan otok šape pacova [246].

Slično tome, levosimendan je takođe ublažio hipoksijom izazvano oštećenje mozga kod pacova ublažavanjem oksidativnog stresa i inflamacije kao i sepsom izazvanu srčanu disfunkciju suzbijanjem oksidativnog stresa, inflamacije kao i regulacijom srčane mitofagije [254, 255]. Najviše studija sa ovakvim modelom I/R lezije je analiziralo antioksidativne efekte kroz praćenje nivoa markera oksidativnog stresa. Tako su alopurinol i dantrolen u kombinaciji smanjili nivoe ROS-a i lezije mukoze creva [55]. Astaksantin je primenjen u istom modelu I/R lezije takođe smanjio nivoe tkivnog MDA, koji je deo TBARS-a, povoljno je delovao na aktivnosti SOD, CAT, smanjio je nivoe proinflamatornih citokina uključujući IL-6 koji je meren i u ovoj studiji. Dodatno je dokazana i inhibicija apoptoze kroz smanjenje aktivnosti CC3 i P53 (deo NF-kB) i smanjenje DNK fragmentacije. Svi ovi nalazi pokazuju da i levosimendan ima antioksidativne mehanizme delovanja kao i ove navedene antioksidativne supstance [256]. U literaturi se sreću brojne studije sa modelom I/R lezije koje su analizirale antioksidativne efekte kroz praćenje nivoa markera oksidativnog stresa i nitrogenog stresa (najčešće NO) kroz aktivnost iNOS enzima [257-259].

Levosimendan smanjuje nivo NO u serumu u odgovoru na inflamaciju kroz smanjenje aktivnosti iNOS promotora u makrofagima, srčanim fibroblastima i hepatocitima u IR leziji jetre [260]. Interesantno je da levosimendan različito deluje na NF-kB u različitim tkivima- nije uticao na aktivaciju i nuklearnu translokaciju i na vezivanje NF-κB za DNK u koloniji J774 makrofaga, ali jeste uticao kao inhibitor na NF-κB- transkripciju u L929 fibroblastima [261]. Iste efekte kao i levosimendan imale su i neke druge supstance kada se analizira NO_2. Tako je, na primer, zaštitni učinak resveratrola značajno pojačan smanjenjem proizvodnje

iNOS-a i NO, što ukazuje na to da ovaj lek, poput levosimendana, ostvaruje svoj zaštitni učinak na oštećenja creva I/R putem NF-κB posredovanog iNOS puta [262].

Lezija intestinalnog zida je dobro poznata posledica AMI pri čemu su crevne resice vrlo osetljive na ishemiju, a epitelna nekroza predstavlja jednu od najranijih histoloških promena. Smatra se da prve promene nastaje već 15 minuta nakon ishemije pre bilo kakvih promena u serumu [20,160]. Parks i Granger su pokazali da reperfuzija nakon ishemije uzrokuje značajno veće oštećenje crevne sluznice nego sama ishemija, što ukazuje na to da hipoksija prvenstveno izaziva lezije sluznice tokom ishemije, dok ROS i RNS doprinose dodatnom oštećenju tokom reperfuzije. Ovi autori su dokazali u svom pionirskom radu iz 1986 godine, da ishemija u trajanju od 4 sata uzrokuje manje štete nego ishemija u trajanju od 3 sata uz jedan sat reperfuzije. Tada je po prvi put pokazano da ponovno uspostavljanje krvotoka i reperfuzija tkiva može da uzrokuje više štete u nivou mikrovaskulature. Ovo istraživanje je pokazalo da hipoksija indukuje lezije mukoze a ROS uzrokuju druge lezije nakon obnove krvotoka [263].

Chiu skor, koji se koristi za procenu stepena oštećenja creva-kvantifikuje stepen histološkog oštećenja creva i pruža mikroskopske dokaze o disrupciji epitela crevnog zida koji idu od najblažeg stepena (0) do najtežeg (V) (0– Normalne crevne resice; I – Proširenje subepitelnog sloja, obično pri vrhu resica, sa kapilarnom kongestijom, II – Proširenje subepitelnog prostora sa umerenim podizanjem epitelnog sloja, III – Masivno podizanje epitela uz bočne ivice resica, IV - Ogoljene resice sa laminom proprijom, izložene proširene kapilare, povećana celularnost lamine proprije, V - Dezintegracija lamine proprije, krvarenje i ulceracije). [264]. Analiza ovog skora pokazala je da pretretman levosimendanom značajno ublažava oštećenje sluznice I/R, što ukazuje na njegovu zaštitnu ulogu tokom faze ishemije i reperfuzije. U grupi koja je dobila pretretman levosimendanom bilo je značajno manje narušavanje arhitektonike crevnih resica, (manja degeneracija), diskretnija je bila zapaljenska reakcija (manja infiltracija ćelija) i bilo je manjeg edema epitela i krvarenja.

Goblet (peharaste) ćelije su vitalne za održavanje strukturalnog i funkcionalnog integriteta crevne barijere, proizvodeći sluz (mucin 2, MUC2). MUC2 je glikoprotein velike molekulske težine koji formira zaštitni nerastvorljivi sloj na površini epitelnih ćelija i koji štiti tanko crevo od bakterijske translokacije i inflamacije, posebno nakon I/R lezije [265]. Ovaj sloj predstavlja fizičku prepreku za bakterije, luminalne toksine i ROS. Ishemija narušava mukusnu barijeru, smanjuje broj goblet ćelija i slabi imunohistohemijsku ekspresiju MUC2

[266]. Povećana sekrecija goblet ćelija i složena egzocitoza brzo deluju protiv toga, oslobađajući uskladištenu sluz kako bi se zaštitile i očistile crevne kripte od bakterijske invazije [267-269]. Međutim, ovo dovodi do smanjenja gustine goblet ćelija. Alciansko plavo bojenje pokazalo je da pretretman levosimendanom povećava gustinu goblet ćelija, čime se štiti sekretorna funkcija tankog creva nakon I/R lezije. U nekim crevnim kriptama je nedostajalo goblet ćelija, što ukazuje da zavisno od trajanja intestinalne I/R lezije potpuni oporavak najčešće nije bio moguć, ali je bio značajan. Održavanje rezervi goblet ćelija da mogu ponovo da luče nove količine MUC2, pošto se obnovi krvotok je od velikog značaja u tretmanu I/R lezije creva, jer to može da znači reverzibilnost promena, to jest da crevo može da se obnovi [269].

Levosimendan deluje antiinflamatorno na smanjenje nivoa IL-6 preko suprimiranja NF-kB [261]. I u studijama na modelima I/R lezije miokarda levosimendan pokazuje isti efekat. Poznato je da ROS aktiviraju NF-kB i da proinflamatorni citokini doprinose inflamatornom odgovoru u mukozi creva gde su makrofagi i neutrofili glavni izvori ovih citokina. NF-kB menja propusnost creva i pojačava leziju creva [270]. U normalnim okolnostima, kada je mukoza creva zdrava, makrofagi lučenjem inhibitora, kao što je IL-10, obezbeđuju homeostazu za mikrobiotu, komensalne bakterije i samog domaćina. Intestinalni makrofazi su inače u stanju mirovanja, ali imaju jak baktericidni potencijal [271]. Kada se aktiviraju bakterijskim LPS-om ili ishemijom oslobađaju citokine, ROS indukuju MPO i dolazi do peroksidacije i potom do fagocitoze. Subpopulacije makrofaga su locirane u mukozi, submukozi i mišićnom sloju crevnog zida i nazivaju se rezidentni makrofagi. U I/R leziji je pokazano da makrofagi značajno povećavaju nivo IL-6 [272]. IL-6 je proinflamatorni 19-26 kd protein koji produkuju makrofagi, fibroblasti, keratinociti i endotelijalne ćelije u odgovoru na stres i brzo se oslobađa tokom reperfuzije. IL-6 nije tkivno specifičan marker i interesantno je da se produkuje tokom eksperimentalnog klemovanja SMA, oslobađa se ubrzo nakon reperfuzije i pojavljuje se rano u cirkulaciji. Ovo oslobađanje IL-6 je delimično pod uticajem TNF [272,273]. U eksperimentima sa ponavljanom ishemijom i reperfuzijom najbrži porast IL-6 je zabeležen nakon prvog puštanja kleme sa SMA [274]. U kliničkoj praksi to se viđa u stanjima ubrzo nakon operacije aneurizme torakalne i abdominalne aorte ili nakon transplantacije bubrega, već u prvih pola sata nakon operacije [275]. U prospektivnoj studiji na pacijentima kod kojih je dokazan AMI nivo IL-6 u serumu je bio značajno veći nego kod pacijenata sa drugim abdominalnim, hirurškim stanjima [276].

Subpopulacija CD68 makrofaga se često koristi u humanim studijama, naročito za istraživanje inflamatorne bolesti creva(*Inflammatory Bowel Disease*, IBD). Nađeno je da je infiltracija crevnog zida CD68 makrofagima značajno veća u akutnoj fazi bolesti u poređenju sa crevom koje nije inflamirano ili je u fazi remisije [277]**.** Primena infliksimaba, monoklonskog antitela značajno je poboljšala zarastanje mukoze kod enteritisa upravo smanjenjem infiltracije CD68 makrofaga u mukozu. U studiji je zapaženo da infliksimab indukuje i apoptozu aktiviranih CD68 makrofaga, što bi mogao da bude značajan terapijski pristup u budućnosti [278]. Snažni antiinflamatorni efekat levosimendana je potvrđen u ovoj studiji. Levosimendan je značajno smanjio infiltraciju makrofaga, ekspresiju IL-6 i NF-kB u crevu pacova koje je bilo zahvaćeno I/R lezijom.

Ranije je spomenuto da se apoptoza povećava sa dužinom trajanja I/R lezije. Ovi rezultati su u skladu s ranijim studijama koje su izveštavale o zaštitnim efektima levosimendana na markere oksidativnog stresa i histološka oštećenja tokom I/R lezije [161, 226, 279, 280]. Neke supstance kao što je estradiol pokazuju slične efekte kao i levosimendan tokom I/R lezije u smislu smanjenja crevne disfunkcije kao što je smanjenje propusnosti crevne barijere i porasta endotelijalne NOS, a takođe i smanjenja markera inflamacije, što u celini smanjuje SIRS [281].

I/R lezija značajno povećava broj apoptotskih ćelija, povećava nivo BAX-a i nivo CC3 a smanjuje ekspresiju antiapoptotskog markera BcL2 u tkivu koje je zahvaćeno ishemijom [282]. Afolabi je u svojoj studiji došao do zaključka da se smanjenjem I/R indukovane aktivacije BAX / CC3 signalizacije može postići oporavak intestinalne barijere i smanjenje bakterijske translokacije [249]. U studiji Zhu-a i saradnika NF-κB ekspresija u crevu tokom I/R lezije je bila značajno manja kod miševa koji su imali mutaciju za TLR 4. Ovo pokazuje da bi blokada TLR4 receptora i signalnog puta TL4 -TNF alfa -NF-kB mogla biti klinički korisna procedura u smanjivanju tkivnog oštećenja nakon intestinalne I/R lezije [283]. Otkako je NF-kB otkriven kao glavni put apoptoze, ispitivan je u različitim tipovima ćelija uključujući i intestinalne epitelne ćelije. U ovoj studiji je evaluirana i ekspresija CC3 kao egzekutorske kaspaze, efektorske proteaze u apoptotskoj kaskadi, koja se često istražuje u modelima I/R lezije. NF-κB je odlična meta za antiinflamatorni tretman, jer sa smanjenjem NF-kB dolazi i do smanjenja proinflamatornih markera [284]. Ovi isti mehanizmi se nalaze i u studijama u kojima su istraživani efekti levosimendana. U studiji Sakagučija i saradnika, zaštitne efekte levosimendan je postigao na humanim hepatocitima delujući supresivno na NF-kB pri čemu je lezija jetre izazvana upotrebom lipopolisaharida (*Lypopolysaccharide,*

LPS) [285]. Levosimendan je na tkivo jetre delovao stimulišući BcL2 (antiapoptotoski protein) i inhibirajući BAX (propapoptotski protein), što je pokazao Bruner u svojoj studiji [211].

Kada se ordinirao sa vazopresorima u standardnoj dozi, tokom izdašne nadoknade tečnosti tokom resuscitacije, levosimendan nije poboljšao srčanu, renalnu i jetrenu funkciju u eksperimentalnom modelu endotoksemije, što se može protumačiti da edem tkiva verovatno ometa njegovu kliničku efikasnost [286]. NF-kB je povezan sa homeostazom i menja propusnost slojeva intestinalnog zida (oštećenjem čvrstih spojeva utiče da paracelularna propusnost raste) te pojačava oštećenje creva [211, 247]. U jednoj od najnovijih studija, levosimendan je smanjio infiltraciju inflamatornih ćelija, fibrozu i ishemijsku nekrozu i u studiji koja je analizirala uticaj levosimendana na zarastanje anastomoze u ishemijskom crevu, što može da ima veliki klinički značaj u abdominalnoj hirurgiji u ranom postoperativnom toku [287]. Levosimendan je značajno smanjio oslobađanje i porast interleukina, NO-a i makrofagnih inflamatornih proteina kao i matriks metaloproteinaze (MMPs) u BALF-u tretiranih životinja u odnosu na grupu životinja koja je bila izložena ventilacijskoj leziji pluća. Treba naglasiti da je u ovom eksperimentu levosimendan bio primenjen u inhalacionom obliku [288].

Podaci iz ove studije pokazuju da pretretman levosimendanom smanjuje nivo NF-kB i to značajno u odnosu na I/R grupu bez pretretmana levosimendanom u kojoj je zabeleženo da aktivacija NF-kB igra značajnu ulogu u odgovoru na leziju terminalnog ileuma tokom rane faze reperfuzije koja je usledila nakon privremene mezenterijalne ishemije. Snažna antiinflamatorna svojstva levosimendana su potvrđena, budući da je pretretman ovim lekom pored smanjenja NF-kB, značajno smanjio i infiltraciju CD68 makrofaga i ekspresiju IL-6 u intestinalnom tkivu pacova nakon I/R lezije.

Model intestinalne I/R lezije koji uključuje 30 minuta ishemije uz 90 minuta reperfuzije je široko korišćen u eksperimentalnim studijama. Ovaj model je razmatran kao pogodna aproksimacija kliničke slike AMI. Iako kompletna ishemija i potom reperfuzija kod ljudi zahteva 6-8, nekada i više od 10 sati [7], brži metabolizam kod pacova (otprilike 6.4 puta brži), više otkucaja srca (4,7 puta više),veći broj respiracija (6,3 puta) i veća izmena proteina (9.6 puta) čini da je 30 minuta ishemije i 90 minuta reperfuzije relevantan prikaz realne urgentne kliničke situacije [289].

Tokom intestinalne I/R lezije, uočen je značajan porast procenta apoptotskih ćelija, što je obeleženo povišenim nivoima cepane kaspaze (CC3) i smanjenom ekspresijom

antiapoptotskog proteina Bcl-2 u zahvaćenim tkivima. Brojne studije su potvrdile da je CC3 ključni proapoptotski faktor koji se aktivira sledeći ishemiju i inicira apoptozu dovodeći do ćelijske smrti [290].

U skladu s ovim nalazima, u ovoj studiji imunohistohemijska analiza je otkrila najveći intenzitet imunoreaktivnosti CC3 u I/R grupi u poređenju s kontrolnom grupom. Važno je napomenuti da je pretretman levosimendanom smanjio nivoe CC3, koja je glavni egzekutor apoptoze, što ukazuje na zaštitni, antiapoptotski učinak tokom I/R povrede. U modelu peritonitisa izazvanog ligacijom cekuma kod pacova, tretman levosimendanom značajno je smanjio ekspresiju CC3 proteina u zahvaćenim tkivima, što je dovelo do pretpostavke da bi jedna rana doza levosimendana mogla biti obećavajuća terapijska strategija za sprečavanje disfunkcije organa povezane s I/R lezijom i sepsom [201]. U modelu ligacije i punkcije cekuma, autori su primenom levosimendana postigli smanjenje ROS-a, IL-1b, iNOS, CC3, smanjenje lezije udaljenih organa, najviše jetre i bubrega, čime su dokazali antioksidativna, antiinflamatorna i antiapoptotska svojstva ovog leka. U istoj studiji levosimendan je poboljšao preživljavanje za 22% u odnosu na kontrolnu grupu [224]. Mnoge studije koje su koristile intestinalne, jetrene i srčane I/R modele pokazale su da pretretman levosimendanom značajno smanjuje DNK fragmentaciju, smanjuje apoptozu, manji je apoptotski indeks (AI), što dokazuje smanjeni broj TUNEL-pozitivnih ćelija [280, 291, 292]. Nalazi ove studije su u skladu s prethodnim istraživanjima, pokazujući da je pretretman levosimendanom značajno smanjio broj TUNEL-pozitivnih epitelnih ćelija u crevnom tkivu. Balans između apoptoze i mitoze, to jest ćelijske proliferacije u nivou mukoze gastrointestinalnog trakta je ključan u I/R leziji i oporavku tkiva [293]. Poremećaj ovog balansa može da dovede ili do slabog odgovora u smislu imunosupresije ili preteranog odgovora kada se govori o razvoju autoimunih poremećaja. Ključni aspekt intestinalne I/R lezije jeste porast broja apoptotskih ćelija u lumenu creva. Nakupljanje apoptotskih ćelija može da dovede do sekundarne nekroze jer iz ovih ćelija mogu isticati citokini i enzimi koji mogu da pojačaju inflamaciju i da izazovu dalje tkivno oštećenje [294, 295]. Grosini i saradnici su u svojoj studiji pokazali da levosimendan deluje protiv I/R lezije kroz mehanizme povezane sa NO produkcijom i aktivacijom mitohondrijskih kalijum zavisnih ATP kanala, na taj način utiče na bolju prokrvljenost perifernih tkiva (inodilatatorna svojstva) a posledično smanjuje i apoptozu [195]. Apoptotske ćelije mogu da se pokrenu pomoću CC3 kroz spoljašnji put ili unutrašnji, takozvani mitohondrijski put koji se aktivira ROS-om, citokinima i NO u većim koncentracijama. Sepsa se skoro sigurno javlja u kasnoj fazi mezenterijalne I/R lezije kada se

moraju intravenski ordinirati visoke doze antibiotika. Levosimendan povećava protok kroz venu porte, protok kroz arterijski mezenterijalni sistem čime poboljšava crevnu mukoznu oksigenaciju i reaktivnost krvnih sudova u eksperimentalnom modelu sepse što može da popravi ishod lečenja [296, 297]. Ovo ukazuje da bi kombinacija levosimendana i antibiotika kao pretretman, mogla da ima važnu ulogu u tretmanu AMI.

Keap1-Nrf2-ARE ((Kelch-like ECH-Associating protein 1) signalni put je jedan od najvažnijih mehanizama odbrane od oksidativnog stresa tokom I/R lezije. Ovaj faktor reguliše ekspresiju nekoliko drugih faktora zaduženih za odbranu ćelija od oksidativnog stresa i inflamacije (citoprotekcija), uključujući HO-1. Nrf2 je glavni činilac u održavanju ravnoteže sluznice ograničavanjem prekomerne proizvodnje ROS-a i zaštitom od inflamacije i oštećenja sluznice putem svojih antioksidativnih efekata [298]. Ovaj faktor podržava preživljavanje i proliferaciju ćelija regulisanjem redoks homeostaze, metabolizma lekova (detoksikacija i izlučivanje ksenobiotika), autofagijom i popravkom DNK [299]. Nrf2 kontroliše antioksidanse poput SOD, CAT, GSH i HO-1, ključne za redoks ravnotežu i ćelijsku homeostazu. Istovremeno, Nrf2 smanjuje aktivaciju inducibilne sintaze azot oksida (iNOS) i aktivnost protein kinaze C kako bi se smanjili nivoi ROS-a i povećali nivoi GSH, čime se ublažava oksidativni stres [247, 300, 301]. Brojne eksperimentalne studije su pokazale značajnu ulogu ovog faktora tokom I/R lezije koja uključuje ishemiju, reperfuziju, inflamaciju i apoptozu. Ovo je veza između signalnih puteva i endogenih antioksidativnih mehanizama odbrane enzimskih SOD i CAT i neenzimskih, kao što je GSH [302].

Brojni su antioksidativni agensi koji aktiviraju antiapoptotske i antiinflamatorne gene regulisane Nrf2 signalnim putem i koji istovremeno inhibiraju proapoptotske i proinflamatorne gene regulisane NF-kB signalnim putem. Eksperimentalne životinje sa deficitom Nrf2 (Nrf2 nokaut miševi) su značajno podložnije citokinima posredovanoj inflamaciji od životinja bez mutacije a koje su bile izložene inflamaciji primenom LPS-a [303]. Slično istraživanjima u kojima su korišćene životinje sa mutacijom za NF-kB i u istraživanjima o Nrf2 i HO-1 signalnom putu tokom I/R rađeno je sa životinjama koje su imale mutaciju- što je dovelo do Nrf2 deficita. Katada i saradnici su tretirali tri grupe životinja (životinje sa mutacijom za Nrf2, sa mutacijom za Bach1, i životinje bez mutacije) supstancom-molekulom koja otpušta ugljen monoksid (*Carbon Monoxide Releasing Molecule*, CORM-3). Bach1 (*BTB domain and homolog1*) je antagonist Nrf2. I/R lezija je izazvana kod svih životinja i uočeno je nakon davanja CORM3 da su inflamatorne promene značajno manje kod životinja sa mutacijom Bach1, kod kojih je Nrf2 bio u suficitu i došlo je do

antiapoptotskog i antiinflamatornog odgovora. Značajno izražene inflamatorne promene su bile u grupi životinja sa mutacijom za Nrf2, koje su imale deficit Nrf2. Ovaj nalaz jasno ukazuje da Bach1 deficijencija dovodi do smanjenja nivoa NF-kB i povećanja Nrf2, dok Nrf2 i HO-1 deficit povećava ekspresiju NF-kB i posledično ostale prooksidativne, proinflamatorne i proapoptotske efekte [304].

Osim antagonističkih odnosa Nrf2 i NF-kB imaju i agonistički, često i sinergistički odnos- sarađuju u održavanju homeostaze i ćelijskog redoks stanja u cilju regulacije odgovora na stres i inflamaciju kako ne bi došlo do preteranog odgovora [94,95].

U studiji Abdel Hamida i saradnika rađena je analiza imunoreaktivnosti Nrf2 i NF-kB u crevnom tkivu. Protektivni efekti (na oksidativni stres, inflamaciju i apoptozu) primenjene supstance-angiotenzin (1-7) su poništeni blokadom receptora i delimično poništeni Nrf2 inhibitorom. Ovo ukazuje na značaj Nrf2 signalnog puta u intestinalnoj I/R leziji [305].

Prema rezultatima ove studije, levosimendan smanjuje oštećenja u crevu tokom I/R lezije na isti način- aktivacijom Nrf2 i HO-1 sistema i inhibicijom NF-kB i egzekutorske CC3. Levosimendan deluje citoprotektivno kroz smanjenje oksidativnog stresa, jačanjem endogenog antioksidativnog odgovora i rebalansom između NF-kB posredovane inflamacije i Nrf2 posredovane antioksidativne zaštite u homogenatu terminalnog ileuma tokom mezenterijalne I/R lezije, slično prethodno navedenim studijama. Slične zaštitne efekte levosimendan je pokazao i u modelu neuroinflamacije, aktivacijom Nrf2/HO-1 puta i istovremenom inhibicijom NF-kB/NLRP3 inflamazom signalnog puta [306].

Inhibicija NF-kB levosimendanom tokom mezenterijalne I/R lezije je postignuta u studiji Polata i saradnika [226]. Hem oksigenaza je samolimitirajući enzim koji katalizuje razgradnju hema do biliverdina, slobodnog gvožđa i ugljen monoksida (CO). Inducibilni oblik ovog enzima pruža citoprotekciju. NF-kB je takođe samolimitirajući enzim- kraj aktivacije se dešava kada novosintetisani IkB ograničava transkripcionu aktivnost NF-kB (negativna povratna sprega), što je naveo Romero sa saradnicima u svojoj studiji o aktivaciji i inhibiciji ovog signalnog puta i uticaju na antioksidativne i antiinflamatorne procese. Autori sugerišu da NF-kB brzo dovodi do skoka nivoa proinflamatornih citokina što takođe može da utiče na ograničenje aktivnosti NF-kB [307].

Povećana ekspresija Nrf2 i HO-1 u grupi koja je prethodno tretirana levosimendanom potvrdila je njegov zaštitni učinak protiv oštećenja creva tokom I/R lezije. Ovo je dodatno

potkrepljeno međudelovanjem između Nrf2 i NF-κB, gde Nrf2 inhibira NF-κB signalizaciju i smanjuje ekspresiju proinflamatornih citokina, čime se smanjuje inflamacija i apoptoza [308-310]. Ovi nalazi su u skladu s prethodnim studijama koje pokazuju blagotvorne učinke levosimendana, koji značajno pojačava Nrf2 signalizaciju u cerebralnim [254], kao i kod bubrežnih i plućnih I/R povreda [291], srčanim [292] i jetrenim I/R lezijama [195].
Uloga levosimendana u tretmanu akutne srčane insuficijencije je dobro poznata. Kao nekateholamisnki inotrop levosimendan ne povećava potrošnju $_c$AMP-a i kiseonika u kardiomiocitima i zato je postao odlična opcija u tretmanu kardiogenog šoka [311]. Otvaranje K_{ATP} kanala sa unutrašnje strane mitohondrija bilo je povezano sa kardioprotekcijom, smanjenjem veličine infarkta i slabljenjem I/R lezije u animalnim eksperimentalnim modelima i u kliničkim studijama. Levosimendan uzrokuje porast minutnog volumena srca i smanjenje plućnog kapilarnog pritiska pri čemu ovi efekti nisu udruženi sa porastom potrošnje energije u miokardu [174,312]. Neke studije zaključuju da levosimendan smanjuje lipidnu peroksidaciju i apoptozu posle I/R lezije i za vreme akutnog srčanog popuštanja [313]. Rezultati iz ove studije su u skladu sa rezultatima ranije sprovedenih studija koje pokazuju protektivni efekat na kardiomiocite kroz smanjenje apoptoze [203]. Ovo je takođe potvrđeno i u kliničkim situacijama kada je levosimendan poboljšao ishode nakon operacije aortokoronarnog bajpasa [177].

Ima malo podataka oko efekta levosimendana na akutnu plućnu leziju [314,315]. Neke studije pokazuju da primena levosimendana uzrokuje smanjenje apoptoze u ćelijama plućnog parenhima [314-316]. Rezultati ove studije su isti sa nalazima ovih studijama.

Nekoliko eksperimentalnih studija na animalnim modelima je pokazalo da levosimendan pored toga što poboljšava srčanu funkciju, dilatira renalne arterije i poboljšava protok krvi kroz bubrege. Ovaj efekat dovodi do smanjenja lezija bubrežnog tkiva što je uočeno imunohistohemijskim analizama [212, 216]. U ovoj studiji reno-protektivni efekat je takođe potvrđen. Ovaj povoljni efekat levosimendana je bio nešto slabije izražen u odnosu na srce i pluća.

Analiza relativne genske ekspresije je dodatno pokazala da levosimendan ima povoljan uticaj na obnovu antioksidativne aktivnosti enzima SOD i CAT. Zapažen je pad u aktivnosti ovih enzima u I/R grupi u odnosu na kontrolnu grupu, dok je zabeležena značajno veća genska ekspresija ovih antioksidativnih enzima u grupi koja je imala pretreman levosimendanom

(LS+I/R) u odnosu na kontrolnu grupu, što jasno pokazuje jak antioksidativni efekat levosimendana tokom akutne ishemije mezenterijuma kod pacova.

Ovo je jedna od prvih studija gde su prikazani efekti levosimendana na akutnu mezenterijalnu I/R leziju kroz analizu markera oksidativnog stresa, inflamacije, patohistoloških i imunohistohemijskih promena i ključnih signalnih puteva. Nove studije koje analiziraju primenu levosimendana ukazuju da je interesovanje za ovu temu i dalje prisutno. Kombinacija antioksidativnog (Nrf2/HO-1), antiinflamatornog (NF-κB/IL-6/CD68) i antiapoptotskog (TUNEL/CC3) efekta levosimendana rezultira sveobuhvatnom zaštitom crevne sluzokože i udaljenih organa tokom i nakon I/R. Ovo se manifestuje kroz očuvanje histološke strukture (goblet ćelije, MUC2, Chiu skor), smanjenje oksidativnog i nitrogenog stresa, kontrolu inflamacije i inhibiciju apoptoze. Rezultati sugerišu da levosimendan može biti potencijalni terapijski agens za prevenciju intestinalne I/R povrede kao i u ranoj fazi AMI, pre razvoja ireverzibilnih promena na crevu. Potrebna su dodatna istraživanja da se potvrdi optimalna doza, vremenski okvir primene i eventualni dopunski sistemski efekti.

7. ZAKLJUČCI

Na osnovu rezultata eksperimentalne studije, mogu se izvesti sledeći zaključci:

Levosimendan je pokazao antioksidativne, antiinflamatorne i antiapoptotske efekte tokom AMI.

1. **Antioksidativni efekti**: Levosimendan ordiniran kao pretretman je smanjio markere oksidativnog stresa. Značajno je smanjen nivo TBARS-a, H_2O_2, NO_2^- i O_2^-, uz povećanu aktivnost antioksidanasa CAT i GSH i SOD u lizatu eritrocita i bronhoalveolarnom fluidu, dok nema efekta na povećanje aktivnosti SOD u homogenatu terminalnog ileuma pacova.

2. **Protektivni efekti na terminalnom ileumu:** Levosimendan je smanjio leziju epitela creva i poboljšao obnovu goblet ćelija.

3. **Antiinflamatorni efekti:** Levosimendan je smanjio faktore inflamacije. Manja je infiltracija CD68 makrofaga i nivo IL-6 u terminalnom ileumu pacova.

4**. Antiapoptotski efekti**: Levosimendan je smanjio apoptotski indeks, smanjio je broj TUNEL pozitivnih ćelija crevnog epitela. Levosimendan je uticao na smanjenu ekspresiju proapoptotskih markera (NF-kB i CC3) i na povećanje ekspresije antiapoptotskih markera-signalnih puteva (Nrf2/HO-1).

5. **Protektivni efekti na udaljenim organima**: Levosimendan je smanjio histopatološki skor oštećenja parenhima srca, pluća i bubrega.

6. Levosimendan je poboljšao relativnu gensku ekspresiju antioksidativnih enzima SOD i CAT u terminalnom ileumu.

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LISTA SKRAĆENICA:

AMI	Akutna mezenterična ishemija
AI	Apoptotski indeks
ASC	Apoptotski protein nalik mrljama koji sadrži domen za regrutaciju kaspaze
ATP	Adenozin trifosfat
aPTT	Aktivirano parcijalno tromboplastinsko vreme
alfa-GST	Serumska alfa-glutation S-transferaza
Bach1	BTB domen i CNC homolog 1
BALF	Bronhoalveolarni ispirak-aspirat
BAX	BCL-2 vezani X protein
BCl-2	B ćelijski limfom-2
BID	BH3 protein poput Bax-a
CAT	Katalaza
CC3	Cepana kaspaza 3
$_c$AMP	Ciklični adenozin mono fosfat
CRP	C-reaktivni protein
DAB	3,3 Diamino benzidin- tetrahidrohlorid
$DAMP_s$	Stimulusi izvedeni iz oštećenih ćelija
DCS	Damage control surgery
DISC	Smrtonosni indukujući signalni kompleks
DMSO	Dimetil sulfoksid
DOAC	Direktni oralni antikoagulansi
EAMI	Embolijska akutna mezenterična ishemija
ESVS	Evropsko društvo za vaskularnu hirurgiju (ESVS)
FADD	Domen smrti vezan za Fas
FasL	Fas ligand

GSH	Redukovani glutation
GST	Glutation S-transferaza
H_2O_2	Vodonik peroksid
HO-1	Hem oksigenaza 1
HRP	Peroksidaza rena
ICAM	Intercelularni adhezioni molekul
IkB	Inhibitor NF kB
I/R	Ishemija/reperfuzija
IL-1	Interleukin 1
IL-6	Interleukin 6
iNOS	Inducibilna azot oksid sintetaza
I-FABP	Protein koji vezuje crijevne masne kiseline
ICG	Indocijanin zelena
ICU	Jedinica intenzivne nege
ICS	Centar za intestinalni udar
IMA	Donja mezenterična arterija
LS	Levosimendan
LMWH	Heparini niske molekularne težine
MAPK	Mitogenom aktiviran put protein kinaze
$_sMAF_s$	Mali muskuloaponeurotični fibrosarkomatozni protein
MDA	Tkivni malondialdehid
MODS	Sindrom multiorganske disfunkcije
MDCTA	Multidetektorska kompjuterizovana tomografija angiografija
MPTP	Mitohondrijalne tranzicione propusne pore
MPO	Mijeloperoksidaza
mtDNA	Mitohondrijska DNK
MVT	Mezenterična venska tromboza

MUC2	Mucin 2
MyD88	Gen primarnog odgovora diferencijacije ćelija 88
NaOH	Natrijum hidroksid
NF-kB	Nuklearni faktor kapa beta pojačivač lakog lanca aktiviranih B ćelija
Nrf2	Nuklearni faktor- eritroid 2 povezani faktor
NO2	Azot dioksid
NLRP3	NOD-like receptor proteina
NOMI	Neokluzivna mezenterična ishemija
O_2^-	Superoksid anjon radikal
·OH	Hidroksil radikal
$ONOO^-$	Peroksinitrit.
$PAMP_s$	Stimulusi izvedeni iz različitih patogena.
RNS	Reaktivne azotne vrste
ROS	Reaktivne kiseonične vrste
SIRS	Sindrom sistemskog inflamatornog odgovora
SMA	Gornja mezenterična arterija
SMV	Gornja mezenterična vena
TAMI	Trombotska akutna mezenterična ishemija
TBARS	Reaktivne vrste tiobarbiturne kiseline
TLR	Tol lajk receptor- naplatni receptor
TRADD	Domen smrti povezan sa TNF receptorom 1
TRAIL	Ligand koji indukuje TNF-vezanu apoptozu
TC	Celijačno stablo
WSES	Svetsko društvo za urgentnu hirurgiju
VCAM	Vaskularni adhezioni molekul
VL	Lijenalna vena
VKA	Antagonisti vitamina K

BIOGRAFIJA:

Ime i prezime: Zoran (Milan) Matković

Datum rođenja: 24.9.1975.

Mesto rođenja: Smederevo

Adresa: Nemanjina 24/5, Doboj

Broj telefona: 065 740-459

Osnovna škola ”Radica Ranković” Lozovik, od 1982 do 1990 godine. Nosilac Vukove diplome i učenik generacije.

Srednja medicinska škola ”Dulic dr Vojislav” Požarevac od 1990 do 1994 godine. Nosilac Vukove diplome i učenik generacije.

Medicinski fakultet Univerziteta u Beogradu od oktobra 1994 do oktobra 2000 godine. Prosečna ocena 9.03.

Obavezan lekarski staž u trajanju od godinu dana - Klinički centar Srbije od novembra 2000 do novembra 2001.

Služba hitne medicinske pomoći Zdravstvenog centra Požarevac od 2002 do aprila 2003.

Javna zdravstvena ustanova Opšta bolnica”Sveti apostol Luka” Doboj od 2003 godine do danas, u službi hirurgije.

Specijalizacija iz opšte hirurgije - Opšta bolnica ”Sveti apostol Luka“ Doboj i Prva hirurška klinika Kliničkog centra Srbije od 2004 do 2010 godine. Ispit položen sa odličnim uspehom.

Član komore doktora medicine Republike Srpske, udruženja hirurga Republike Srpske, udruženja endoskopskih hirurga u BiH. Član Evropskog udruženja endoskopskih hirurga (EAES). Zvanje primarijus od aprila 2021 godine. Rukovodilac odseka abdominalne hirurgije u matičnoj ustanovi, od 2022 godine. Oženjen, otac troje dece.

Прилог 3.

Изјава 1

ИЗЈАВА О АУТОРСТВУ

Изјављујем
да је докторска дисертација

Наслов рада „Ефекти левосимендана у акутној исхемији мезентеријума код пацова"

Наслов рада на енглеском језику „Effects of levosimendan in acute mesenteric ischemia in rats"

- ☒ резултат сопственог истраживачког рада,
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У Бањој Луци, дана 22.01.2026. године

Потпис докторанта

Изјава 2

Изјава којом се овлашћује Универзитет у Бањој Луци
да докторску дисертацију учини јавно доступном

Овлашћујем Универзитет у Бањој Луци да моју докторску дисертацију под насловом
„Ефекти левосимендана у акутној исхемији мезентеријума код пацова"

која је моје ауторско дјело, учини јавно доступном.

Докторску дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у дигитални репозиторијум Универзитета у Бањој Луци могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (*Creative Commons*) за коју сам се одлучио/ла.

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- ( ) Ауторство – некомерцијално – дијелити под истим условима
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(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

У Бањој Луци, дана 22.01.2026. године

Потпис докторанта

Изјава 3

Изјава о идентичности штампане и електронске верзије докторске дисертације

Име и презиме аутора	Зоран Матковић
Наслов рада	„Ефекти левосимендана у акутној исхемији мезентеријума код пацова"
Ментор	Проф.др Зоран Алексић

Изјављујем да је штампана верзија моје докторске дисертације идентична електронској верзији коју сам предао/ла за дигитални репозиторијум Универзитета у Бањој Луци.

У Бањој Луци, дана 22.1. 2026. године

Потпис докторанта

International Journal of
Molecular Sciences

MDPI

Article

Levosimendan Pretreatment Attenuates Mesenteric Artery Ischemia/Reperfusion Injury and Multi-Organ Damage in Rats

Zoran Matković [1], Milica Gajić Bojić [2,3], Uglješa Maličević [2,4], Aleksandra Krivokuća [2,4], Nebojša Mandić-Kovačević [2,5], Snežana Uletilović [2,6], Ljiljana Amidžić [2,7], Sanja Jovičić [2,8], Maja Barudžija [2,8], Miloš P. Stojiljković [2,3], Radoslav Gajanin [2,9,10], Sergej Bolevich [11] and Ranko Škrbić [2,3,10,11,*]

[1] Department of Surgery, General Hospital "Sveti Apostol Luka", 74000 Doboj, Bosnia and Herzegovina; zoranmatna@gmail.com
[2] Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina; milica.gajic@med.unibl.org (M.G.B.); ugljesa.malicevic@med.unibl.org (U.M.); aleksandra.krivokuca@med.unibl.org (A.K.); nebojsa.mandic-kovacevic@med.unibl.org (N.M.-K.); snezana.uletilovic@med.unibl.org (S.U.); ljiljana.amidzic@med.unibl.org (L.A.); sanja.jovicic@med.unibl.org (S.J.); maja.barudzija@med.unibl.org (M.B.); milos.stojiljkovic@med.unibl.org (M.P.S.); radoslav.gajanin@med.unibl.org (R.G.)
[3] Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[4] Department of Pathophysiology, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[5] Department of Pharmacy, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[6] Department of Medical Biochemistry and Chemistry, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[7] Department of Human Genetics, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[8] Department of Histology and Embryology, Faculty of Medicine, University of Banja Luka, 78000 Banja Luka, Bosnia and Herzegovina
[9] Department of Pathology, University Clinical Centre of the Republic of Srpska, 78000 Banja Luka, Bosnia and Herzegovina
[10] Academy of Sciences and Arts of the Republic of Srpska, 78000 Banja Luka, Bosnia and Herzegovina
[11] Department of Pathologic Physiology, First Moscow State Medical University "I.M. Sechenov", 119435 Moscow, Russia; bolevich2011@yandex.ru
* Correspondence: ranko.skrbic@med.unibl.org

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Abstract

Acute mesenteric ischemia (AMI) is a life-threatening condition characterised by oxidative stress, inflammation, apoptosis, and necrosis of intestinal epithelial cells. Different drugs with vasoactive, antioxidant, and anti-inflammatory properties have been used to treat AMI. Levosimendan is a drug with proven anti-ischemic effects used in the management of acute congestive heart failure. This study evaluated the protective effects of levosimendan pretreatment on intestinal, as well as lung, heart, and kidney tissue in a rat model of mesenteric artery ischemia/reperfusion (I/R) injury. Male Wistar rats (N = 24) were divided into four groups: control, I/R, levosimendan (LS) 1 mg/kg i.p, and LS + I/R (1 mg/kg i.p. 30 min before injury). I/R by itself caused elevation of oxidative markers (thyobarbituric acid reactive species (TBARS), hydrogen peroxide (H_2O_2), super oxide anjon radical (O_2^-), and nitrogen dioxide (NO_2^-)), induced inflammation (macrophage infiltration and Interleukin-6 (IL-6) production), and apoptosis (nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), cleaved caspase-3 (CC3), and terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)). Levosimendan pretreatment significantly reduced oxidative stress markers and enhanced antioxidant defences (catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD)). Histological analysis

revealed reduced mucosal damage and preserved goblet cells in intestinal tissue. Similar protective effects of levosimendan were observed in other organs such as lung, heart, and kidney. Immunohistochemistry showed reduced epithelial apoptosis and upregulation of antioxidant and anti-inflammatory proteins. These findings highlight levosimendan's ability to protect mesenteric I/R tissue injury and multi-organ damage by suppressing oxidative stress, inflammation, and apoptosis, emphasising its therapeutic potential in clinical settings.

Keywords: ischemia/reperfusion; mesenteric artery; levosimendan; oxidative stress; inflammation; apoptosis

1. Introduction

AMIis defined as insufficient perfusion in the mesenteric vascular bed caused by inadequacy of the arterial supply or venous drainage of the intestine [1]. The interrupted blood flow is followed by intestinal ischemia and tissue oedema with further progression of intestinal gangrene, manifested as peritonitis, multi-organ dysfunction syndrome (MODS), cardiovascular collapse, and death. Although its incidence is low (6.2 per 100,000 inhabitants), AMI is a potentially fatal vascular, gastrointestinal, and surgical condition with a high mortality rate of 60–80% [2,3]. Despite the progress in the understanding of pathogenesis and development of modern treatment modalities, AMI remains a clinical challenge [4,5]. The current treatment options, like the early administration of anticoagulants, vasodilators, and thrombolytic therapy, are primarily oriented toward revascularisation and restoration of mesenteric blood flow [6]. It was found that the restoration of circulation and the release of reactive oxygen species (ROS) cause even greater damage to the intestine than the previous ischemia itself [2,7]. While mesenteric ischemia disturbs the function of intestinal villi, reperfusion following blood flow restoration exacerbates ischemic damage that would induce intestinal necrosis. This can raise the risk of bacterial translocation and sepsis, as well as the risk of MODS, which is the main cause of death in patients with mesenteric I/R injury [8].

Intestinal (I/R) injury involves various cascades like production of ROS, mitochondrial alteration, intestinal permeability disturbance, activation of innate immune response, and production of pro-inflammatory mediators leading to induction of apoptotic signalling pathways [8]. The activation of innate immune response following mesenteric I/R enhance toll-like receptor 4 (TLR4)/ NF-κB pathway and subsequently alleviate both systemic and intestinal levels of tumour necrosis factor alpha (TNF-α) and IL-6 [9,10].

According to therapeutic guidelines, the treatment of AMI includes different conservative procedures, such as intravenous rehydration, anticoagulant, analgetic, thrombolytic, and antiplatelet therapy. These could be combined with additional surgical protocols, including endovascular arterial revascularisation or bowel resection [6,11].

Different procedures and drugs have been used to attenuate AMI. Drugs with vasoactive, antioxidant, and anti-inflammatory effects have been investigated in experimental models of AMI [12]. It has been shown that milrinone and levosimendan can improve the perfusion of the primary area of ischemia, without effect on the splanchnic vasoconstriction [12,13]. Levosimendan is a drug with proven anti-ischemic effects, attributed to numerous pleiotropic effects, including antioxidant, anti-inflammatory, and anti-apoptotic effects. As a calcium sensitiser in the cell, levosimendan leads to greater cardiac muscle fibre strength without increasing energy expenditure, as reflected by its anti-ischemic activity. Additionally, levosimendan is a powerful vasodilator through the opening of adenosine

triphosphate (ATP) -dependent potassium channels in the smooth muscle cells [13]. Apart from the myocardium, levosimendan causes vasodilation in other organs, including the lungs, intestines, liver, and kidneys. Its active metabolite, OR 1896, with a very long half-life of 80 h, is responsible for the prolonged beneficial effect [14]. However, levosimendan is not used in the treatment of patients with AMI, and it has been rarely used even in preclinical studies with experimental AMI models [15,16].

The aim of this study was to investigate the protective effects of levosimendan on intestinal mucosa and multi-organ damage, following mesenteric I/R injury in rats through evaluation of oxidative stress, tissue inflammation, and apoptosis.

2. Results

2.1. Levosimendan Attenuates the Effects of Mesenteric I/R Injury on Oxidative Stress Markers in Blood, Intestinal Tissue, and Bronchoalveolar Lavage Fluid

A significant increase in the lipid peroxidation index (TBARS) was observed in I/R group compared to the control. This notable increase in plasma TBARS was associated with elevated levels of all the tested pro-oxidative markers, like H_2O_2, O_2^-, and NO_2 ($p < 0.05$). The I/R group also showed a decrease in levels of antioxidative enzymes CAT, SOD, and GSH, as measured in erythrocyte lysate. However, pretreatment with levosimendan mitigated the effects of mesenteric I/R injury, as evidenced by a significant reduction in plasma levels of TBARS and pro-oxidative enzymes ($p < 0.05$). Additionally, pretreatment with levosimendan exhibited an antioxidant effect by increasing the levels of antioxidant enzymes, SOD, CAT, and GSH, compared to I/R group (Figure 1).

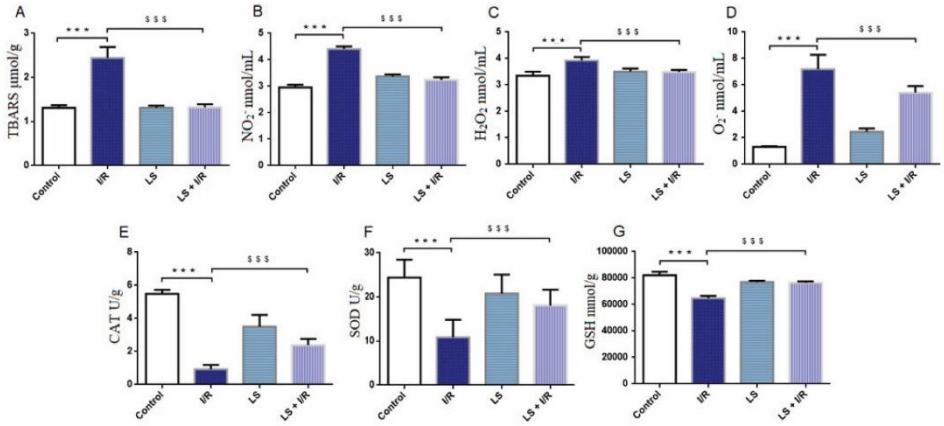

Figure 1. Effects of levosimendan (LS) on oxidative stress markers in rat plasma and erythrocyte lysate. (**A**) TBARS (µmol/g); (**B**) NO_2^- (nmol/mL); (**C**) H_2O_2 (nmol/mL); (**D**) O_2^- (nmol/mL); (**E**) CAT (U/g); (**F**) SOD (U/g); (**G**) GSH (mmol/g). Mesenteric I/R injury led to a significant increase in the lipid peroxidation index (TBARS) and pro-oxidative markers: H_2O_2, NO_2^-, and O_2^-, along with a decrease in antioxidant enzymes (CAT, SOD, and GSH), while pretreatment with levosimendan significantly reduced plasma TBARS and pro-oxidative markers and enhanced antioxidant enzyme levels compared to the I/R group. Data are expressed as mean ± SD. Control (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6). *** $p < 0.001$ comparing control with I/R group; $^{\$\$\$}$ $p < 0.001$ comparing I/R with LS + I/R group.

Consistent with plasma findings, TBARS levels were significantly ($p < 0.05$) elevated in terminal ileum tissue homogenates of rats in the I/R group compared to the control group. In addition, CAT, SOD, and GSH activities were significantly decreased in the terminal ileum tissue homogenates of rats subjected to I/R compared to controls ($p < 0.05$). The elevated values of TBARS were restored and antioxidative CAT and GSH activities were

significantly improved in I/R group pretreated with levosimendan ($p < 0.05$). However, levosimendan pretreatment did not demonstrate beneficial effects on SOD activity in ileum tissue homogenate (Figure 2).

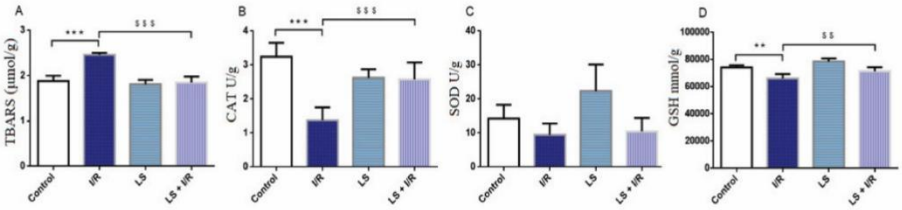

Figure 2. Effects of levosimendan (LS) on oxidative stress markers in rat intestinal tissue homogenate. (**A**) TBARS (µmol/g); (**B**) CAT (U/g); (**C**) SOD (U/g); (**D**) GSH (mmol/g). TBARS levels are significantly increased and CAT, SOD, and GSH activities are significantly decreased in I/R groups compared to controls, while levosimendan pretreatment significantly reduced TBARS levels and improved CAT and GSH activities, but had no beneficial effect on SOD activity. Data are expressed as mean ± SD. Control (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6). ** $p < 0.01$, *** $p < 0.001$, comparing control with I/R group; $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$, comparing I/R with LS + I/R group.

Levosimendan modulated oxidative stress markers in rat bronchoalveolar lavage fluid (BALF). In the I/R group, TBARS ($p < 0.01$) and NO_2^- ($p < 0.001$) levels were markedly elevated, whereas CAT, SOD, and GSH activities were significantly reduced compared to controls ($p < 0.001$). Pretreatment with levosimendan attenuated the increase in TBARS and NO_2^- ($p < 0.001$) and significantly restored CAT ($p < 0.01$), SOD ($p < 0.05$), and GSH ($p < 0.001$) activities toward control levels (Figure 3).

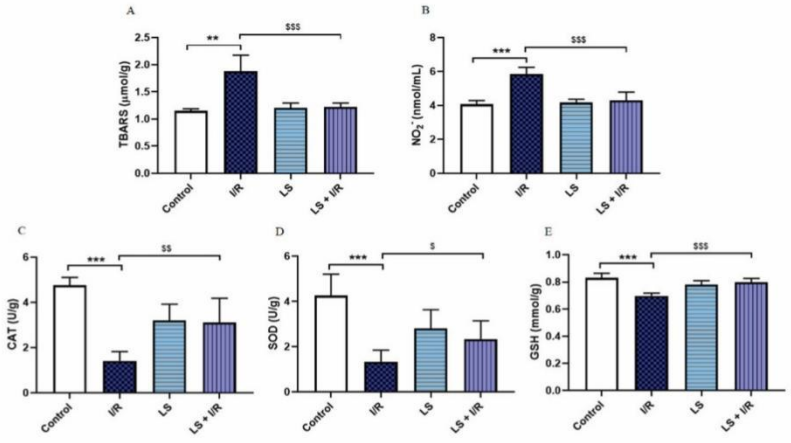

Figure 3. Effects of levosimendan (LS) on oxidative stress markers in rat BALF. (**A**) TBARS (µmol/g); (**B**) NO_2^- (nmol/mL); (**C**) CAT (U/g); (**D**) SOD (U/g); (**E**) GSH (mmol/g). TBARS levels are significantly increased and CAT, SOD, and GSH activities are significantly decreased in I/R groups compared to controls, while levosimendan pretreatment significantly reduced TBARS levels and improved CAT, SOD, and GSH activities. Data are expressed as mean ± SD. Control (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6). ** $p < 0.01$; *** $p < 0.001$ comparing control with I/R group; $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$, comparing I/R with LS + I/R group.

2.2. Protective Effects of Levosimendan on Rat Terminal Ileum Epithelial Cells Induced by I/R Injury

As shown in Figure 3, the ileum sections of the I/R group displayed disruption of the mucosal villi architecture, associated with significant epithelial lifting, inflammatory infiltration, and haemorrhage. The LS group displayed histological findings comparable to

the control. Pretreatment with levosimendan showed significantly less disintegration of the mucosal villi, along with reduced inflammatory infiltration and haemorrhage in the lamina propria, compared to the I/R group. The terminal ileum injury grade, based on the Chiu score, was used to semi-quantify the differences between groups stained by haematoxylin and eosin (Figure 4).

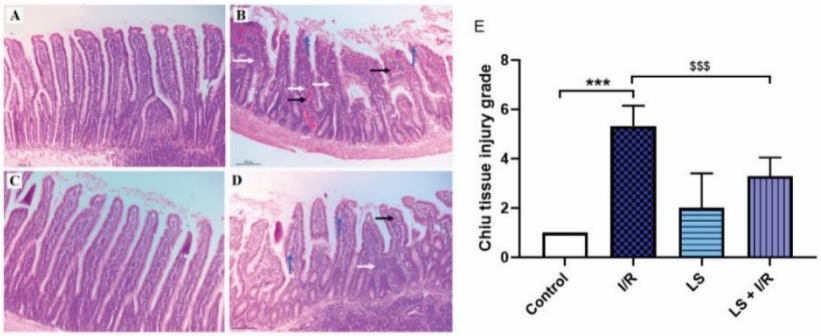

Figure 4. Representative microphotographs of rat terminal ileum sections stained by haematoxylin and eosin (magnification 10×, scale bar = 100 μm). (**A**) Preserved architecture of the terminal ileum mucosal villi in the control group. (**B**) The I/R group displays severe disruption of mucosal villi architecture with extensive epithelial lifting (blue arrows), inflammatory cell infiltration (white arrows), and haemorrhage (black arrows) within the lamina propria. (**C**) LS group showed normal terminal ileum histology. (**D**) The LS + I/R group exhibits notably reduced mucosal damage, with milder inflammatory infiltration and haemorrhage compared to the I/R group. (**E**) Terminal ileum injury was graded according to the Chiu scoring system (mean ± SD, Control (N = 6); I/R (N = 6); LS (N = 6); LS + I/R (N = 6); 10 fields per sample). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (between indicated groups).

The histopathologic examination of the rat terminal ileum stained with Alcian blue revealed findings similar to those previously observed with haematoxylin and eosin staining. The control group shows normal terminal ileum histology with a typical distribution of goblet cells in the crypts of Lieberkühn and the epithelial layer of the mucosa. The I/R group showed destruction of villi, inflammatory infiltrate, and absence of goblet cells in the crypts. In the LS group a normal distribution of goblet cells was seen, while the pretreatment with levosimendan in I/R group had a mostly normal distribution of goblet cells, with occasional absence in some crypts (Figure 5).

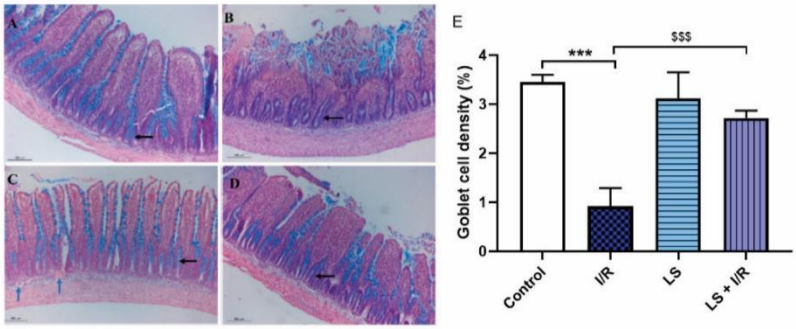

Figure 5. Effects of levosimendan (LS) on histopathology of intestinal tissue in mesenteric I/R injury. Small intestine cross section, magnification ×100 (Alciane blue stain, scale bar 100 μm). (**A**) Control

group shows normal terminal ileum histology, with a typical distribution of goblet cells in Lieberkühn's crypts (black arrow) and epithelial layer of the mucosa. (**B**) I/R group shows destruction of the apical part of villi, inflammatory infiltrate at the base, and absence of goblet cells in crypts (black arrow). (**C**) LS group exhibits a thickened submucosa with dilated blood vessels (blue arrows) and normal mucosa histology with goblet cells in crypts (black arrow). (**D**) LS + I/R group shows dilated villi, mostly normal goblet cell distribution, but occasional absence in single crypts (black arrow). (**E**) Numerical areal density of goblet cells (%), showing significant preservation in the LS + I/R group (mean ± SD, Control (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6); 10 fields per sample). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (between indicated groups).

2.3. Effects of Levosimendan on Inflammatory Response of Intestinal Tissue Induced by Mesenteric I/R Injury

In addition to causing structural damage and depletion of antioxidant defences, mesenteric I/R injury resulted in a pronounced elevation of inflammatory mediators. CD68 and IL-6 immunoreactivity indicated their key involvement in initiating and sustaining inflammation in I/R injury. In the control group, only a small number of CD68-positive macrophages and IL-6-positive cells were observed in the stromal region outside the blood vessels within the villi. In contrast, the I/R group showed a significant increase in CD68-positive macrophages and IL-6-positive cells, predominantly localised at the erosion fronts of the damaged villi. The levosimendan group showed immunoreactivity comparable to the control group, whereas levosimendan pretreatment significantly reduced macrophage infiltration and IL-6 expression in I/R-injured tissue (Figure 6).

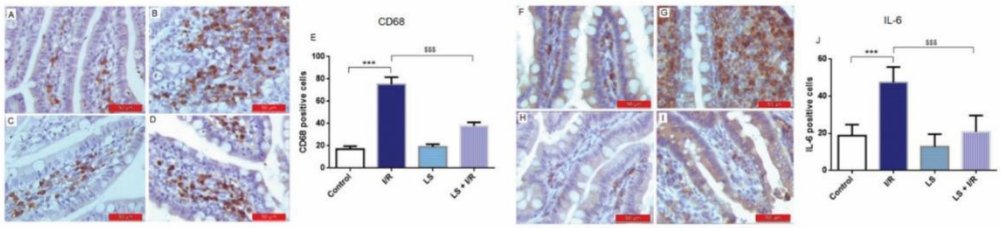

Figure 6. Immunohistochemical results of the expression of CD68 (left panel) and IL-6 (right panel) in rat small intestinal tissue. Data are expressed as mean ± SD. Representative immunohistochemical images at 400× magnification. (**A**,**F**) Control groups. (**B**,**G**) A marked elevation in the number of CD68-positive macrophages (**B**) and IL-6-positive cells (**G**) was observed in the I/R groups. (**C**,**H**) LS group exhibited similar immunoreactivity to the control. (**D**,**I**) The LS + I/R group exhibited significantly decreased immunoreactivity for both macrophages and IL-6. (**E**,**J**) Percentage of diaminobenzidin (DAB) -stained cells in small intestine tissue indicating the proportion of CD68-positive (**E**) and IL-6-positive (**J**) cells. *** $p < 0.001$ comparing control with I/R group; $^{\$\$\$}$ $p < 0.001$ comparing I/R with LS + I/R group.

2.4. Levosimendan Attenuated Apoptosis of Intestinal Epithelial Cells Induced by I/R Injury

Immunohistochemistry analysis revealed a significant upregulation of NF-κB and CC3. NF-kB and CC3 in the I/R group, indicating that these markers play a key role in the pathophysiological response to rat ileum damage and apoptosis. Pretreatment with levosimendan (1 mg/kg) significantly attenuated intestinal epithelial cell apoptosis induced by I/R injury, as evidenced by downregulation of NF-kB and CC3 expression. The results showed that levosimendan pretreatment could alleviate I/R-induced intestinal injury (LS + I/R vs. I/R, $p < 0.001$) (Figure 7).

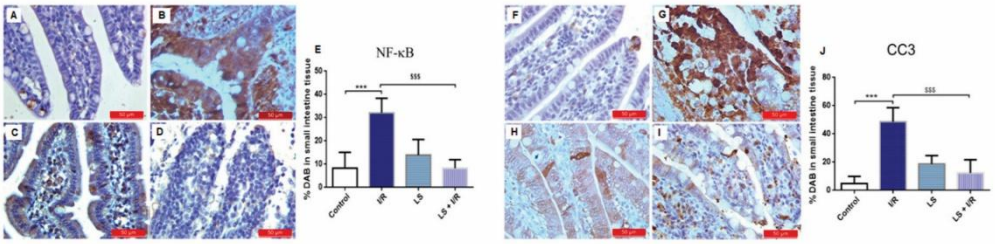

Figure 7. Immunohistochemical results of the expression of NF-kB (left panel) and CC3 (right panel) in rat small intestinal tissue. Data are expressed as mean ± SD. Representative immunohistochemical images at 400× magnification. (**A**,**F**) Control groups. (**B**,**G**) Intense cytoplasmic staining of NF-κB (**B**) and CC3 (**G**) in intestinal epithelial cells of the I/R groups, indicative of apoptosis-related activation. (**C**,**H**) LS groups showed no significant difference in immunoreactivity to apoptosis markers compared to the control groups. (**D**,**I**) Marked reduction in epithelial apoptosis and intestinal damage in the LS + I/R groups. (**E**,**J**) Percentage of DAB-stained cells in small intestine tissue indicating the proportion of NF-κB-positive (**E**) and CC3-positive (**J**) cells. *** $p < 0.001$ comparing control with I/R group; $^{\$\$\$}$ $p < 0.001$ comparing I/R with LS + I/R group.

To further confirm the anti-apoptotic effects of LS, a TUNEL analysis (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling) was performed. Levosimendan pretreatment decreased apoptotic index, which was observed as a significantly decreased number of mostly TUNEL-positive epithelial cells of intestinal tissue (Figure 8).

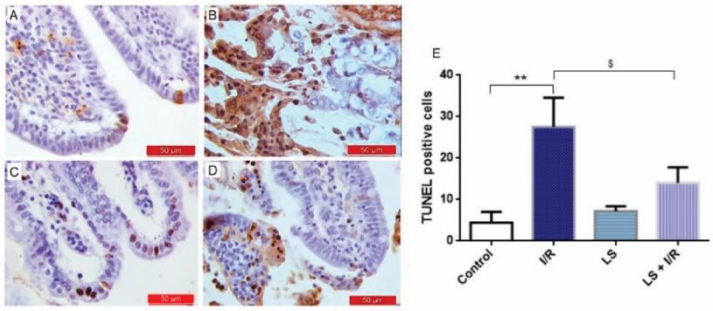

Figure 8. Levosimendan inhibited apoptosis in rat small intestinal tissue in mesenteric I/R injury detected by TUNEL staining, magnification 400×. (**A**) Control group. (**B**) A significant increase in DNA fragmentation was observed in the I/R group, evidenced by intensified brown TUNEL staining. (**C**) The LS group exhibited a similar appearance and distribution of TUNEL-positive cells as the control group. (**D**) Pretreatment with levosimendan prevented I/R-induced nuclear apoptosis. (**E**) Quantitative analysis of apoptotic cells was performed on immunohistochemically stained sections of rat intestinal tissue. ** $p < 0.01$ comparing control with I/R group; $^{\$}$ $p < 0.05$ comparing I/R with LS + I/R group.

2.5. Levosimendan Pretreatment Induced Upregulation of Nrf2, HO-1, and Nrf2/HO-1 Signalling

Furthermore, the results indicate that overexpression of nuclear factor erythroid 2-related factor (Nrf2), as well as the hem oxygenase 1 (HO-1) antioxidant response in the terminal ileum reduce intestinal I/R injury. The control and levosimendan pretreated groups showed similar appearance and distribution of Nrf2- and HO-1-positive cells, with no significant differences observed, whereas the I/R group exhibited a marked decrease in immunoreactivity. Levosimendan successfully elevated the levels of these antioxidant markers, as evidenced by significant differences between the LS + I/R and I/R groups (Nrf2, $p < 0.01$; HO-1, $p < 0.001$) (Figure 9).

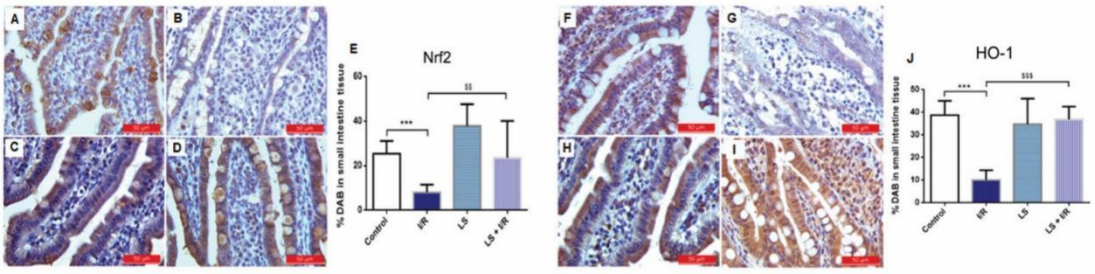

Figure 9. Immunohistochemical results of the expression of Nrf2 (left panel) and HO-1 (right panel), in rat small intestinal tissue. Data are expressed as mean ± SD. Representative immunohistochemical images at 400× magnification. (**A**,**F**) Control groups. (**B**,**G**) Marked reduction in immunoreactivity for the antioxidant markers Nrf2 (**B**) and HO-1 (**G**) was observed in intestinal epithelial cells of the I/R group. (**C**,**H**) Immunoreactivity for both markers in the LS group was comparable to that observed in the control group, with no significant differences detected. (**D**,**I**) Pretreatment with levosimendan significantly increased antioxidant marker levels compared with the I/R group. (**E**,**J**) Percentage of DAB-stained cells in small intestine tissue indicating the proportion of Nrf2-positive (**E**) and HO-1-positive. (**J**) cells. *** $p < 0.001$ comparing control with I/R group; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$ comparing I/R with LS + I/R group.

2.6. Protective Effects of Levosimendan on Heart, Lung, and Kidney Induced by Mesenteric Artery I/R Injury

I/R injury induces damage not only in thegastrointestinal tract but also in remote organs such as the heart, lungs, and kidneys. The myocardial injury is most pronounced in the septal region and apex of the heart. Histopathological examination reveals cardiomyocyte hypertrophy, extensive inflammatory infiltrates predominantly composed of lymphocytes, and consequent myocardial necrosis. Levosimendan administration demonstrates significant vasodilatory properties, as observed by the presence of dilated blood vessels engorged with erythrocytes within the endomysium. Pretreatment with levosimendan confers marked cardioprotection, reflected by improved tissue damage scores. Cardiomyocyte integrity is preserved, vascular dilation in the endomysium persists, and only sparse inflammatory cells remain evident (Figure 10).

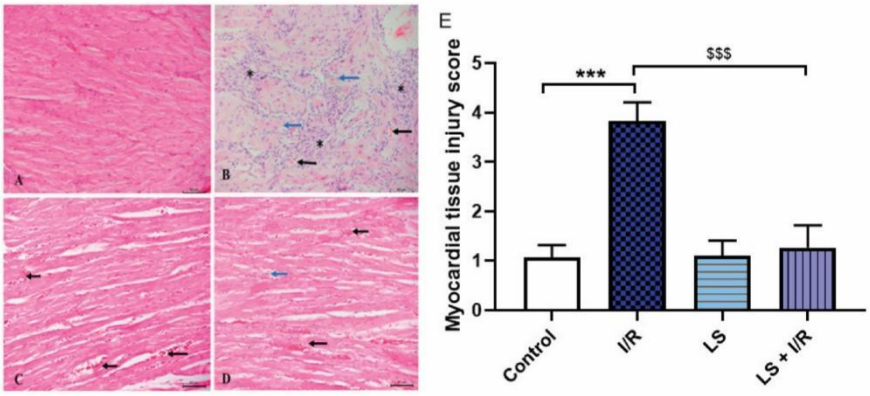

Figure 10. Representative microphotographs of rat cardiac muscle longitudinal sections stained with haematoxylin and eosin (magnification ×20, scale bar = 50 µm). (**A**) Preserved histological structure of the cardiac muscle in the control group. (**B**) The I/R group displays dense inflammatory infiltrate permeating the cardiac muscle (asterisk); presence of damaged cardiomyocytes (blue arrow) and erythrocyte extravasation (black arrow). (**C**) LS group preserved histological structure of the cardiac

muscle, with dilated blood vessels in the endomysium (black arrow). (**D**) The LS + I/R group preserved histological structure of the cardiac muscle; in addition to dilated blood vessels in the endomysium (black arrow), scattered inflammatory cells are also present (blue arrow). (**E**) Tissue damage score of cardiac muscle (mean ± SD, Control, N = 6; LS, N = 6; I/R, N = 6; LS + I/R, N = 6). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (between indicated groups).

The lungs are among the organs most severely affected by I/R injury. This condition leads to diffuse damage of the pulmonary parenchyma, accompanied by significant impairment of the respiratory epithelium. Nearly all alveoli exhibit structural damage and are infiltrated with dense inflammatory cells. Additionally, fibrotic changes are evident in the basal regions of the lungs. Pretreatment with levosimendan confers substantial protection to the pulmonary parenchyma, with only sparse inflammatory cell foci persisting in the basal lung areas, while the majority of the pulmonary tissue remains preserved (Figure 11).

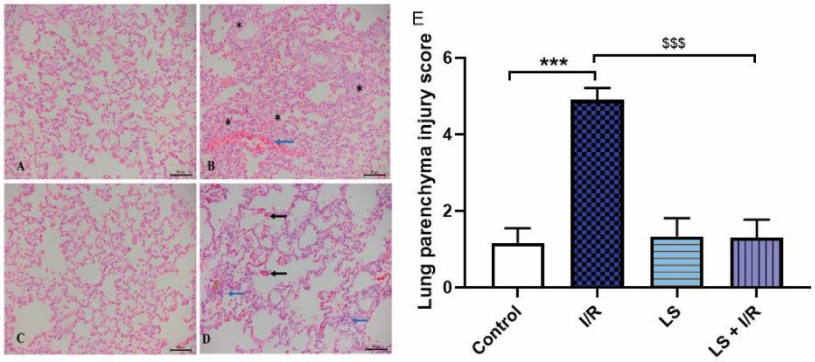

Figure 11. Representative microphotographs of rat lung parenchyma sections stained with haematoxylin and eosin (magnification ×20, scale bar = 50 µm). (**A**) Preserved histological structure of the lung parenchyma in the control group. (**B**) The I/R group displays severe damage, with interalveolar spaces completely filled with inflammatory infiltrate (asterisk), dilated blood vessels are present, and erythrocyte extravasation (black arrow). (**C**) LS group preserved histological structure of the lung parenchyma. (**D**) The LS + I/R group mostly preserved histological structure of the lung parenchyma; scattered inflammatory cells (blue arrow) and erythrocyte extravasation (black arrow) are present. (**E**) Tissue damage score of lung parenchyma (mean ± SD, Control (N = 6), I/R (N = 6), LS (N = 6), LS + I/R (N = 6); 10 fields per sample). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (between indicated groups).

The renal parenchyma, including both the cortex and medulla, is also susceptible to damage during mesenteric I/R injury. Dense inflammatory infiltrates permeate the cortical region, causing complete degeneration of the glomeruli. Although pretreatment with levosimendan demonstrates a protective effect on the renal parenchyma, tissue damage score analysis reveals that its protective impact is relatively limited in this tissue compared to other organs. Histological examination shows preservation of the glomeruli; however, epithelial damage persists in the collecting ducts within the medullary region, accompanied by sparse foci of inflammatory cells (Figure 12).

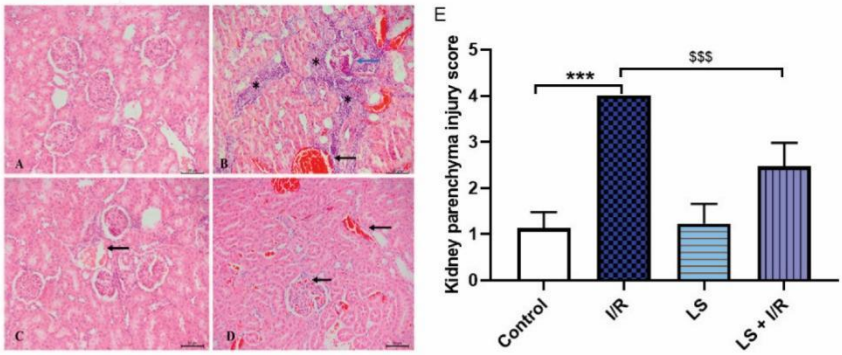

Figure 12. Representative microphotographs of rat renal cortex sections stained with haematoxylin and eosin (magnification ×20, scale bar = 50 µm). (**A**) Preserved histological structure of the renal cortex in the control group. (**B**) The I/R group displays dense inflammatory infiltrate (asterisk) in renal parenchyma; completely damaged renal corpuscle (blue arrow), dilated blood vessels with erythrocyte extravasation (black arrow). (**C**) LS group preserved histological structure of the renal cortex, slightly dilated blood vessel (black arrow). (**D**) The LS + I/R group preserved histological structure of the renal cortex; presence of dilated blood vessels, including glomerulus blood vessels (black arrow). (**E**) Tissue damage score of renal parenchyma (mean ± SD, Control (N = 4), I/R (N = 6), LS (N = 5), LS + I/R (N = 6); 10 fields per sample). *** $p < 0.001$; $^{\$\$\$}$ $p < 0.001$ (between indicated groups).

3. Discussion

In this study, we found that levosimendan pretreatment exhibits antioxidant, anti-inflammatory, and anti-apoptotic properties that are responsible for multi-organ tissue protective effects in an experimental model of mesenteric artery I/R injury in rats.

Intestinal mucosa tissue is very sensitive to hypoxia and a high amount of oxygen is required to maintain the functional integrity of the digestive tract. When AMI occurred, the oxygen deprivation initiated the intracellular blockade of mitochondrial oxidative phosphorylation, with consecutive ATP depletion and inhibition of ATPase-dependent ionic pumps. This inevitably induces the increase in intracellular sodium and calcium levels and disintegration of cytoskeleton and cellular membranes, which can finally lead to cell rupture and necrosis, particularly if ischemia is prolonged. During ischemia, ATP is gradually converted into adenosine that can be further degraded to inosine and hypoxanthine, which accumulates in the ischemic tissue [8,17].

After the tissue is reperfused, the enzyme xanthine oxidase utilises the freely available oxygen to oxidise hypoxanthine. This process is associated with O_2^- production, which is then transformed by SOD into H_2O_2, and this molecule is further cleaved into highly reactive and cytotoxic hydroxyl radicals (·OH). Additionally, O_2^- can combine with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$). The excessive production of reactive oxygen/nitrogen species (ROS/RNS) initiate the fragmentation of proteins and nucleic acids, as well as the lipid peroxidation of cellular membranes. All these events ultimately lead to epithelial cell apoptosis and necrosis [8]. However, a large amount of free oxygen radicals generated during the reperfusion phase cannot be removed by the endogenous antioxidant system (SOD, CAT, and GSH). Therefore, many antioxidant compounds have been investigated for reduction in the damage caused by I/R injury [18,19].

In our study, levosimendan significantly reduced the oxidative stress markers (TBARS, H_2O_2, and NO_2) and increased the activity of antioxidative enzymes (CAT and SOD) and GSH, demonstrating a strong antioxidant effect in serum, intestine homogenate, and BALF. These results are in accordance with previous study, in which levosimendan showed

strong antioxidative properties in carrageenan-induced inflammatory paw oedema of rat [18]. Similarly, levosimendan also alleviated the sepsis-induced cardiac dysfunction by suppressing oxidative stress and inflammation and regulating cardiac mitophagy [20], as well as the hypoxia-induced brain injury in rats by ameliorating the oxidative stress and inflammation [21].

It is known that ROS activate NF-kB and trigger the transcription of many pro-inflammatory mediators like TNF-alfa, IL-6, and IL-1. These cytokines contribute to the inflammatory response in the intestinal mucosa, where neutrophils and macrophages are a major source of these cytokines. NF-kB is linked to homeostasis and alters the permeability of the intestinal layer and intensifies the intestinal damage [22]. The strong anti-inflammatory property of levosimendan was confirmed in our study, since the pretreatment with this drug significantly reduced macrophage infiltration, IL-6, and NF-kB expression in rat intestinal tissue following I/R injury.

Intestinal wall damage is a well-recognised consequence of AMI, with the intestinal villi being highly susceptible to ischemia and epithelial necrosis representing one of the earliest histological changes [10,11]. Parks and Granger demonstrated that reperfusion following ischemia causes significantly greater intestinal mucosal injury than ischemia alone, suggesting that hypoxia primarily induces mucosal lesions during ischemia, while ROS and RNS contribute to additional damage during reperfusion [23,24]. The Chiu score analysis, which is used to assess the degree of intestinal damage, demonstrated that levosimendan pretreatment significantly mitigates mucosal I/R injury, indicating its protective role during both ischemia and reperfusion phases. Goblet cells are vital for intestinal barrier integrity, producing mucus that protects the small intestine from bacterial translocation and inflammation, especially after I/R injury [25]. Ischemia disrupts the mucus barrier, while increased goblet cell secretion and compound exocytosis rapidly counteract this, releasing stored mucus to protect intestinal crypts from bacterial invasion [26–28]. Alcian blue staining showed that levosimendan pretreatment increased goblet cell density, thus preserving the secretory function of the small intestine after I/R injury. These results are consistent with earlier studies reporting the protective effects of levosimendan on oxidative stress markers and histological damage during I/R injury [15,16].

The intestinal I/R injury model involving 30 min of ischemia and followed by 90 min of reperfusion is widely used and considered as a suitable approximation of human clinical conditions. Although complete reperfusion in humans typically takes 6–8 h, the accelerated metabolism in rats (approximately 6.4 times higher) makes a 90 min reperfusion period a relevant reflection of urgent clinical scenarios [29]. During intestinal I/R injury, a significant increase in the percentage of apoptotic cells has been observed, marked by elevated levels of cleaved CC3 and reduced expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) in affected tissues [30]. Numerous studies have confirmed that CC3, a key pro-apoptotic factor, is activated following ischemia and initiates apoptosis, leading to cell death [31,32]. Consistent with these findings, immunohistochemical analysis revealed the highest intensity of CC3 immunoreactivity in the I/R group compared to controls. Importantly, pretreatment with levosimendan reduced CC3 levels, indicating a protective, anti-apoptotic effect during I/R injury. In the rat model of caecal ligation peritonitis, levosimendan treatment significantly reduced CC3 protein expression in affected tissues, leading to the assumption that a single early dose of levosimendan could be a promising therapeutic strategy to prevent organ dysfunction related to I/R injury and sepsis [33,34].

Multiple studies using intestinal, hepatic, and cardiac I/R models have demonstrated that pretreatment with levosimendan significantly reduces the apoptotic index, as evidenced by a decreased number of TUNEL-positive cells [35–37]. The findings of this study are consistent with previous research, demonstrating that levosimendan pretreatment

significantly reduced the number of predominantly TUNEL-positive epithelial cells in intestinal tissue.

Nrf2 is a key transcription factor that maintains mucosal balance by limiting excessive ROS production and protecting against inflammation and mucosal damage through its antioxidant effects [38]. It supports cell survival and proliferation by regulating redox homeostasis, drug metabolism, and DNA repair [39]. Nrf2 controls enzymatic antioxidants like SOD, CAT, GSH, and HO-1, crucial for redox balance and cellular homeostasis, while reducing inducible nitric oxide synthase (iNOS) activation and protein kinase C activity to lower ROS and increase GSH levels, thus mitigating oxidative stress [22,40,41]. Recent studies indicated that Nrf2/HO-1 system activation might become an appropriate strategy to mitigate oxidative stress and inflammation to protect the organs from I/R injury via anti-inflammatory and anti-apoptotic effects [42–44]. The up-regulation of Nrf2 and HO-1 in the levosimendan pretreated group confirmed its protective effect against intestinal I/R injury. This is further supported by the interplay between Nrf2 and NF-κB, where Nrf2 inhibits NF-κB signalling and decreases pro-inflammatory cytokine expression, thereby reducing inflammation and apoptosis [45–47]. These findings are consistent with previous studies demonstrating the beneficial effects of levosimendan, which markedly enhances Nrf2 signalling in cerebral [21] as well as in renal, lung [33], heart [36], and liver I/R injuries [37].

The role of levosimendan in the treatment of acute heart failure is well known. As a non-catecholamine inotrope that does not increase cardiomyocyte cyclic AMP and oxygen consumption, it has become an excellent option for the treatment of cardiogenic shock [48]. Opening of potassium ATP channels of the inner side of mitochondria has been associated with cardioprotection, reduction in infarct size, and attenuation of I/R injury in animal model studies, as well in clinical studies. Levosimendan causes an increase in cardiac output and decrease in pulmonary capillary pressure, which is not accompanied by an increase in myocardial energy consumption [49,50]. Some studies concluded that levosimendan decreased the lipid peroxidation and apoptosis after I/R injury and during severe heart failure [51]. Our results are similar to previous study that showed protective effects of levosimendan on cardiomyocytes [36,52]. This was also confirmed in clinical settings, in which levosimendan improved outcomes after coronary artery bypass [53].

There are limited literal data about the effects of levosimendan on lung injury [54,55]. Some studies have shown that levosimendan administration caused a reduction in the apoptosis of lung tissue cells [54–56]. Our results are consistent with these reports.

Several animal studies have shown that levosimendan, in addition to improving heart function, dilates renal arteries and improves kidney blood flow. This effect leads to a reduction in kidney tissue lesions detected by histological and immunohistochemical analyses [57,58]. In our study the reno-protective effect of levosimendan was also confirmed.

4. Materials and Methods

4.1. Ethical Principles

All procedures were conducted with the approval of the Ethical Committee of the Medical Faculty of Banja Luka, in accordance with guidelines for working with experimental animals and ensuring animal welfare (Approval ID: 18/1. 331-3/23, dated 6 June 2023). Animal treatment was carried out in accordance with the Law of protection and welfare of animals of the Republic of Srpska (Official Gazette of the Republic of Srpska, No 111/08) and the directive of European parliament for conducting experiments on animals (210/63/EU), guidelines of Animal Research: Reporting of in vivo experiments (ARRIVE).

4.2. Experimental Animals and Protocols

Male Wistar albino rats, weighing 280–320 g (8–12 weeks old), were kept under controlled laboratory conditions, at 21 ± 2 °C room temperature, 55 ± 5% humidity, and a 12 h light–dark cycle, with access to food and water "ad libitum". Twenty-four animals were randomly divided into four groups. All animals underwent a midline laparotomy incision under general anaesthesia induced intraperitoneally with a combination of 90 mg/kg ketamine and 10 mg/kg xylazine. The sham group was used as a control (Control group, N = 6). The I/R group was used as the model disease for AMI, with clamping of the SMA for 30 min, followed by 90 min of reperfusion (I/R group, N = 6). The LS group received levosimendan (1 mg/kg. i.p.) and underwent the same sham procedures (LS group, N = 6). The LS + I/R group received levosimendan (1 mg/kg, i.p.) 30 min before the onset of I/R injury (LS + I/R group, N = 6). Rats from the Control and LS groups received DMSO as a solvent for levosimendan (Figures 13 and 14). At the end of the experiment, all animals were sacrificed under deep anaesthesia by exsanguination performed through puncture of the thoracic artery, and blood and organ tissue samples were collected for further analysis.

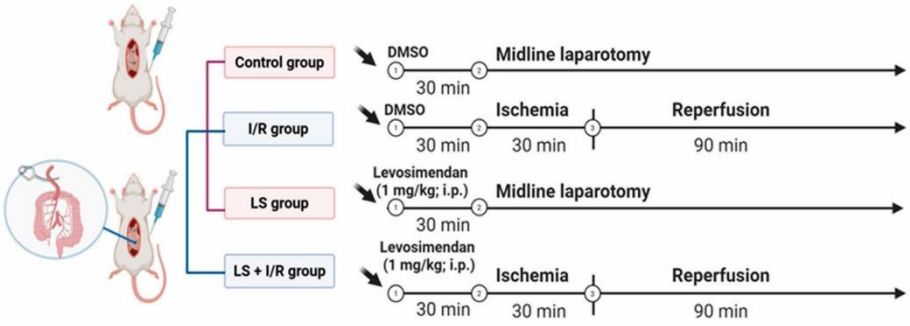

Figure 13. Illustration of the experimental study design. The schematic shows different experimental groups and timing of interventions (drug administration, midline laparotomy, ischemia, and reperfusion). Figure created with BioRender.com.

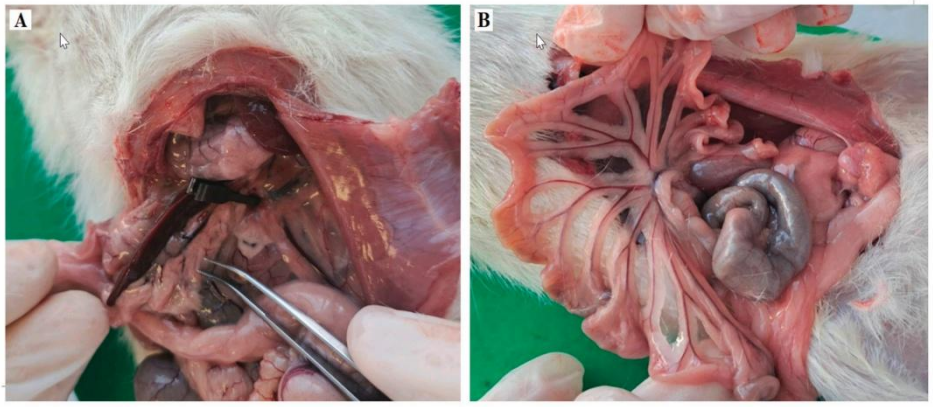

Figure 14. Representative photographs showing (**A**). SMA was exposed by carefully detaching from the surrounding tissue. The isolated SMA was occluded with an atraumatic artery microclamp (bulldog) at its branching from the aorta. (**B**) Ischemia of the intestine was verified by observation of dark discoloration of the intestinal loops (mostly ileum), loss of pulsation in mesenteric vessels, and visible intestinal atony (after 30 min of ischemia). Free peritoneum was protected by using a gauze soaked with warm 0.9% saline to minimise evaporative heat and fluid loss.

4.3. Oxidative Stress Markers

Oxidative stress levels were assessed in plasma, erythrocyte lysate, BALF, and terminal ileum tissue homogenate. Plasma prooxidative markers, including H_2O_2, NO_2^-, and O_2^-, were quantified using the methods of Pick and Keisari [59], the Green method [60], and Nitro Blue Tetrazolium (NBT) reduction method [61], respectively. Lipid peroxidation was evaluated by measuring TBARS using 1% thiobarbituric acid (TBA) and 0.05 M sodium hydroxide (NaOH), with readings taken at 530 nm [62]. Antioxidant levels in erythrocyte lysate, including catalase CAT, SOD, and GSH, were measured spectrophotometrically according to Beutler's methods [63–65].

4.4. Histopathological Examination and Morphometric Analysis

After 48 h of fixation in 4% formaldehyde, tissue samples were processed using a Leica TP 1020 tissue processor and embedded in paraffin blocks. Sections were cut to a thickness of 4 µm with a Rotatory 3003 pfm microtome and stained using routine haematoxylin and eosin staining, as well as with an Alcian Blue Stain kit (pH 2.5, mucin stain, Abcam, Cambridge, United Kingdom, CB2 OAX). The samples were analysed under a Leica DM 6000 binocular microscope equipped with a Leica DFC310FX camera. For morphometric analysis, the LAS V4.12 software was used at ×10 and ×20 magnification, depending on the type of tissue and organ, on 10 visual fields for each sample. Tissue damage score was determined semi-quantitatively as previously published [66–69]. Numerical areal density was determined for tissue slides stained with Alcian Blue. This density reflects the number of goblet cells in the terminal part of the small intestine relative to the entire tissue area. The visual field area was first calculated in square millimetres; then, the numerical areal density (NA) was calculated as the quotient of the number of structures or cells (N) to the area of the visual field (A), with NA = N/A. The resulting values are expressed as percentages.

4.5. Immunohistochemical Analysis

The activation of a specific apoptotic pathway was evaluated using immunohistochemical staining with primary antibodies NF-κB, CC3, Nrf2, and HO-1. Inflammatory markers were assessed by immunohistochemical analysis using primary antibodies to CD68 to confirm the presence of an immune cell infiltrate and ongoing immune response, as well as primary antibodies to IL-6 to identify a cytokine-mediated inflammation. Antibodies were applied to the samples and incubated overnight at 4 °C in a humid chamber. Detection was achieved using secondary antibodies conjugated with horseradish peroxidase (HRP) and a polyvalent detection system (UltraVision Detection System HRP Polymer & DAB Plus Chromogen, Thermo Fisher Scientific, 47777 Warm Springs Blvd. Fremont, CA, USA). Antibody binding sites became visible after staining with the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB), and the samples were subsequently stained using the haematoxylin and eosin method.

Apoptosis was also analysed by the TUNEL method using a commercial TUNEL detection kit (TUNEL In Situ Kit, Elabscience, Wuhan, China; catalogue number: E-CK-A331) according to the manufacturer's instructions. Briefly, paraffin sections were deparaffinised, rehydrated, and treated with proteinase K for permeabilisation. After washing, the tissue was incubated with a reaction solution containing TdT enzyme and labelled nucleotides at 37 °C for 60 min. Streptavidin-HRP conjugate was then added, and the signal was visualised using DAB substrate, with apoptotic cells shown as brown-stained nuclei. Sections were counterstained with haematoxylin, dehydrated, and mounted for light microscopic

analysis. The apoptotic index (AI), representing the percentage of apoptotic cells, was calculated using the following formula:

$$\text{AI (\%)} = (\text{Number of TUNEL-positive cells} \times 100)/\text{Total number of cells}$$

All samples were examined under a Leica DM2500 optical microscope and captured with an MC170HD camera at 400× magnification. Microphotographs were archived in TIFF format. The immune response was analysed using Fiji software (Version 2.14.0/1.54f, National Institutes of Health, Bethesda, MD, USA), focusing on the number of DAB-positive cells and the mean optical density in the positive intestinal tissue. Results are presented as mean optical density ± standard deviation for 10 fields of view per rat, and the average optical density was compared between groups.

4.6. Statistical Analysis

Statistical analysis was performed with IBM-SPSS Statistic version 20.0 software (SPSS, Inc., Chicago, IL, USA), while GraphPad Prism 6.0 software was used for graphical representation. ANOVA test is used to compare the means of parametric characteristics and Kruskal–Wallis is used to compare the nonparametric characteristics between the groups. Tukey and Bonferroni tests are used for "post hoc" analysis. Results are presented as mean ± standard error, and the level of significance was set at $p < 0.05$.

5. Conclusions

Levosimendan pretreatment exerts protective effects in intestinal I/R injury in rats through antioxidative, anti-inflammatory, and anti-apoptotic mechanisms. In our study, levosimendan administration during I/R significantly attenuated oxidative stress, as evidenced by reduced levels of TBARS, H_2O_2, NO_2^-, and O_2^- and with increased activities of CAT and GSH. Histological analysis revealed a lower intestinal injury score and restoration of goblet cells, alongside reduced multi-organ tissue damage. Levosimendan downregulated pro-apoptotic markers NF-κB and CC3, alongside upregulation of the Nrf2 and HO-1 signalling molecules. These findings suggest that the protective effects of levosimendan are closely linked to signalling mechanisms responsible for suppression of pro-oxidative, pro-inflammatory, and pro-apoptotic responses, emphasizing its therapeutic potential in clinical settings.

Limitation of the Study

The findings of this study suggest that levosimendan can mitigate mesenteric I/R injury and subsequent multi-organ damage by reducing oxidative stress, inflammation, and apoptosis. Nevertheless, the exact molecular mechanisms underlying these protective effects remain unclear, as no specific cellular target has yet been identified to account for its antioxidative, anti-inflammatory, and anti-apoptotic actions.

It is also important to note that these benefits were observed only under pretreatment conditions. This raises questions about their clinical relevance, since therapeutic administration after injury is of greater importance than prevention in the context of ischemia-reperfusion.

Consequently, future studies are needed to identify specific cellular targets underlying the molecular mechanisms of levosimendan's mode of action and to evaluate its potential clinical efficacy when administered as a treatment.

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Z.M.; writing—review and editing, R.Š.; visualisation, L.A.; supervision, M.P.S. and M.G.B.; project administration, U.M. and N.M.-K.; funding acquisition, S.J. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

AI	Apoptotic index
AMI	Acute mesenteric ischemia
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
Bcl-2	B cell lymphoma-2
CAT	Catalase
CC3	Cleaved caspase 3
DAB	3,3′-diaminobenzidine tetrahydrochloride
DMSO	Dimethyl sulfoxide
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HO-1	Hem Oxygenase 1
HRP	Horseradish peroxidase
I/R	Ischemia/reperfusion
IL-1	Interleukin-1
Il-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
LS	Levosimendan
MODS	Multiple Organ Dysfunction Syndrome
NaOH	Sodium hydroxide
NF-kB	Nuclear Factor kappa light-chain enhancer of activated B cells
NO_2	Nitrogen dioxide
Nrf2	Nuclear factor erythroid 2- related factor
O_2^-	Super oxide anjon radical
OH	Hydroxyl radical
$ONOO^-$	Peroxynitrite
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROS/RNS	Reactive oxygen/nitrogen species

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REVIEW ARTICLE

Clinical Aspects of Acute Mesenteric Ischaemia

Zoran Matković,[1] Uglješa Maličević,[2, 3] Milica Gajić Bojić,[2, 4] Aleksandra Krivokuća,[2, 3] Đorđe Đukanović,[2, 5] Nataša Đekić Matković,[1] Zoran Aleksić[6]

Abstract

Acute mesenteric ischaemia (AMI) is a serious disease with mortality between 50 and 80 %. Oxidative stress plays a major role in the pathophysiology of AMI. AMI should be considered for any acute abdominal pain that requires analgesia with morphine and for which no other obvious aetiology is found. CT is the main diagnostic procedure to confirm the diagnosis of AMI. There is no specific diagnostic biomarker for AMI that can be used in routine practice. AMI is an urgent diagnostic and therapeutic situation. Treatment of AMI includes a protocol combining digestive rest, curative anticoagulant, antiplatelet, antibiotic therapy, arterial revascularisation to salvage viable bowel and resection of necrotic digestive segments. The strategy of revascularisation depends on the mechanism of arterial occlusion, the morphological appearance of the lesions and the indications for exploratory laparotomy. Endovascular and open surgical techniques can be combined and complemented. Open surgical revascularisation is indicated in case of failure or impossibility of endovascular revascularisation and in case of need for laparotomy. Early diagnosis and timely surgical intervention are the cornerstones of modern treatment to reduce the high mortality of AMI. The emergence of endovascular approaches and modern imaging techniques is developing and providing new treatment options. A multidisciplinary approach based on early diagnosis and treatment is necessary.

Key words: Mesenteric ishaemia, acute; Oxidative stress; Mesenteric vascular occlusion; Diagnostic imaging; CT; Treatment.

1. General Hospital Doboj, Doboj, The Republic of Srpska, Bosnia and Herzegovina.
2. Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina.
3. Department of Pathophysiology, Faculty of Medicine, University of Banja Luka, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina.
4. Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Banja Luka, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina.
5. Department of Pharmacy, Faculty of Medicine, University of Banja Luka, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina.
6. University Clinical Centre of the Republic of Srpska, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina.

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Corresponding author:
ZORAN MATKOVIĆ
E: zoranmatna@gmail.com

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Introduction

Acute mesenteric ischaemia (AMI) represents a pathophysiological state characterised by an inadequacy in splanchnic blood flow, resulting in an insufficient supply to fulfil the metabolic needs of the intestine.[1] The aetiology of AMI encompasses both, arterial and venous origins, contributing to the intricate nature of this vascular disorder. In this article, focus is only on the detailed aspects of AMI caused by artery-related factors. Through an in-depth exploration of its arterial aetiology, aim is to provide comprehensive insights into the pathogenesis, clinical manifestations, diagnostic modalities and management strategies associated with this critical medical condition. According to a retrospective study dating back to 1993, AMI is reported to account for approximately one hospitalisation in 1000 in emergency departments across Europe and the United States. People older than 50 are more likely to develop intestinal ischaemia.[2] Despite this historical perspective, the diagnostic challenges associated with AMI persist, contributing to the likelihood that its true fre-

quency remains substantially underestimated. The comparison of two European studies shows a difference in the incidence of AMI estimated by the usual diagnostic methods (0.63 per 100,000 people per year) and that evaluated using a series of autopsies (12.9 per 100,000 people per year).[3,4]

The mortality of AMI is particularly high, estimated between 50 and 80 %, partly due to the diagnostic difficulty leading to a delay in treatment.[5] In a retrospective study published in 2015 in which 780 cases of AMI treated in intensive care were studied, the mortality rate was 58 %.[6] This was probably even underestimated because the study included left-sided ischaemic colitis, not rare complications of vascular surgery for abdominal aortic aneurysms and whose prognosis is less severe than in small bowel ischaemia.

The advent of expert centres (Intestinal Stroke Centre (ISC), *Structure d urgences vasculaires Intestinales* (SURVI)) in the management of AMI offers hope for improving the prognosis of this pathology.[7,8]

Mechanisms of acute mesenteric ischaemia

AMI can be of occlusive origin, with arterial involvement (85 to 95 %) largely predominating over venous involvement (5 to 15 %) or non-occlusive. AMI linked to an arterial occlusion is most often related to an embolism (40 to 50 %), which should lead to a search for cardiac arrhythmia due to atrial fibrillation, an intracardiac thrombus or an atherosclerotic plaque of the thoracic aorta. Thrombotic occlusion occurring in pre-existing atheromatous stenosis is the second most common mechanism (20 to 35 %). Dissections and vasculitis represent less than 5 % of cases. The incidence of non-occlusive AMI is very poorly evaluated because their diagnosis is difficult. The studies were carried out mainly in selected populations of patients post-operatively for cardiac surgeries or abdominal aortic aneurysms and showed an incidence of 3 to 20 %.[9-11] The pathophysiology of non-occlusive AMI is often linked to a state of shock with low flow associated with diffuse mesenteric vasoconstriction in response to hypovolaemia, reduced cardiac output and/or vasopressor amines necessary for resuscitation.

Pathophysiology of acute mesenteric ischaemia

Splanchnic circulation represents approximately 25 % of resting cardiac output.[12] The physiology of splanchnic flow regulation is complex, involving intrinsic (metabolic and myogenic) and extrinsic (autonomic nervous system and hormonal) regulation systems.[12] The interruption or significant reduction in intestinal blood flow leads to AMI from the mucous layer to the serosa according to a complex multi-step pathophysiological process, which can lead to irreversible transmural necrosis or intestinal infarction, to a multiple organ dysfunction syndrome (MODS) and death in the absence of early and appropriate treatment. Reperfusion exacerbates tissue damage to a greater extent than ischaemia alone.[1,13,14]

Oxidative stress during acute mesenteric ischaemia

In recent years, numerous studies have highlighted the significant role of oxidative stress in the pathogenesis of AMI. During reperfusion, the reintroduction of oxygen leads to an abundance of reactive oxygen species (ROS) within damaged cells and tissues. These ROS can indiscriminately target various intracellular biomolecules including membranes, organelles and DNA fragments, contributing to the progression of tissue damage. This process, oxidative stress, disrupts the dynamic balance (homeostasis) of epithelial cells through signal transmission, which is followed by the release of large amounts of inflammatory mediators and the induction of apoptosis and worsening of damage during and after reperfusion.[15] Mitochondrial DNA participates in oxidative phosphorylation of the cells and maintains normal cell function. After mitochondrial DNA damage, the production of ROS increases and this DNA is released into the cytoplasm and proinflammatory and proapoptotic factors are activated.[16] ROS mainly come from the gastrointestinal tract, pathogens can produce inflammatory factors by activating epithelial cells, polymorphonuclear cells and macrophages.[17] ROS in small and moderate quantities are useful for physiological processes, but in large excess, they can lead to oxidative tissue damage.[18] These include compounds such as superoxide anion (O_2-), hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2). Beside this, nitrogen oxide (NO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$)

are collectively named reactive nitrogen species, which are closely related to ROS and are often listed together. The common feature of these radicals is that they contain unpaired electrons and that they are highly reactive toward intracellular proteins, lipids and even DNA.[19] Under physiological conditions, ROS are neutralised by endogenous antioxidative enzymes and are not harmful to the body.[20] The mitochondrial respiratory chain regulates the production of ROS. Increased ROS generation cause the mPTP (mitochondrial permeability transition pore) to open and release apoptotic factors into the cytoplasm (Figure 1).[21] Enzymes that generate ROS *in vivo* conditions, also include lipooxygenases, glucose oxidases, nitric oxide synthetases and cyclooxygenase activation.[22] AMI can induce hypoxia, triggering the irreversible conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). This conversion process generates ROS, contributing to tissue damage (Figure 1).[23] After the reperfusion starts and oxygen supply is renewed (oxygen wave), the electrons from XO are transferred to molecular oxygen creating significant amounts of oxygen free radicals such as O_2-, OH-, H_2O_2.[24] Intestinal ischaemia-reperfusion injury can reduce the height of intestinal villi, increasing cellular infiltration and worsening the peeling of intestinal mucosa observed histologically. In addition, proinflammatory cytokines (TNF, IL-1, IL-6) are released into the serum and lead to systemic disturbances such as systemic inflammatory response syndrome (SIRS) and MODS.[25] The endogenous antioxidants can somewhat protect cells and tissue from ROS attacks. Enzymatic antioxidants: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR) and heme oxygenase (HO) and nonenzymatic antioxidants such as glutathione (GSH), thioredoxin (TRX), melatonin play important role in oxidative stress homeostasis.[26]

Development of MODS during AMI

Several hypotheses have been proposed to explain the occurrence of MODS in cases of acute intestinal distress.[27, 28] The changes in the lining of the digestive system and the lymphoid tissue linked with it, known as gut-associated lymphoid tissue (GALT), along with their interaction with the normal bacteria in the intestines appear to be significant factors in causing MODS.[28, 29] The breakdown of tight junctions in the gut lining al-

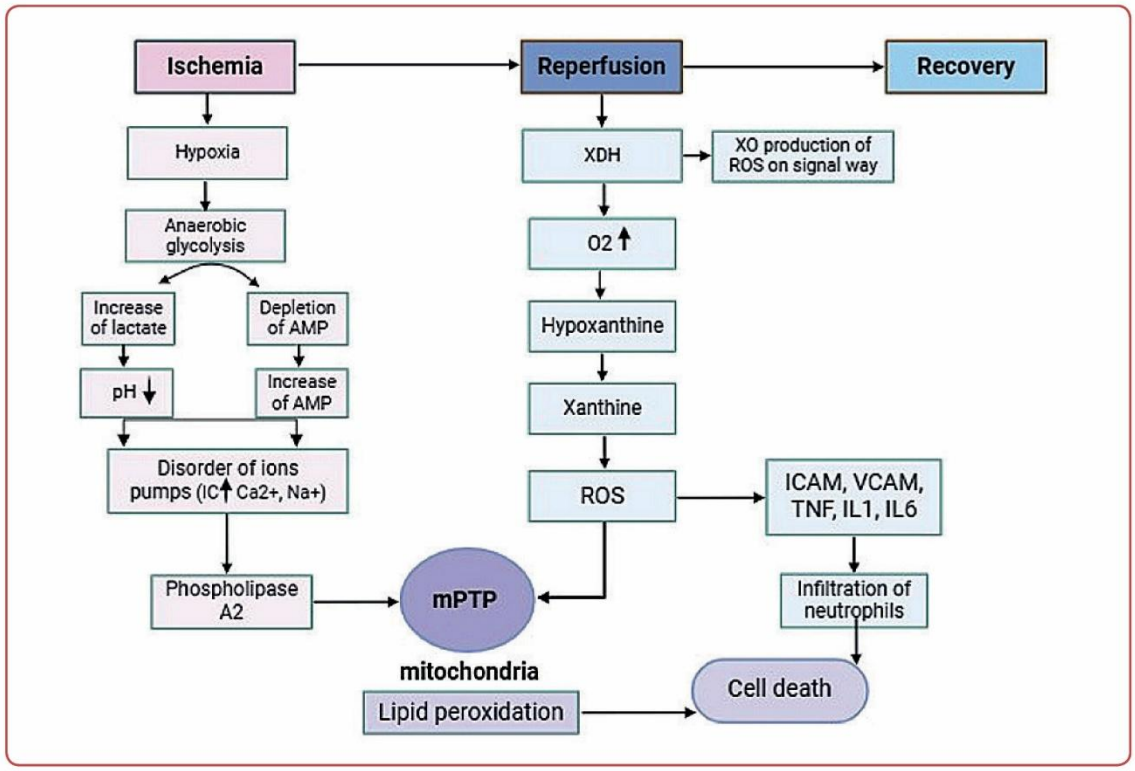

Figure 1: Patophysiologic mechanisms involved in acute mesenteric ischaemia (AMI)

lows harmful substances like bacteria, toxins and inflammatory molecules to pass into the body. Additionally, during acute intestinal distress, about half of the immune cells in the gut wall decrease due to a process called apoptosis. This weakens the body's ability to fight off pathogens from the gut, making it easier for them to enter the bloodstream and cause further problems.[30] Microbial overgrowth, mainly anaerobic, from commensal flora at the site of injured intestine due to paralytic ileus, contributes to the risk of bacterial translocation.[31, 32]

To understand the possible role of AMI in the occurrence of systemic complications such as acute respiratory distress syndrome, liver and kidney lesions, the systemic passage of pathogens from the intestinal lumen was studied in the mesenteric lymph nodes, lymphatic circulation and blood portal venous. In six clinical studies involving 2,125 patients undergoing laparotomy, primarily for cancer surgery rather than AMI, it was found that bacterial translocation into the mesenteric lymph nodes occurred in 5 to 21 % of cases. This phenomenon was linked to a higher likelihood of postoperative infectious complications.[8, 33-37] In one study that involved 20 patients with severe abdominal trauma, among whom 30 % developed MODS, an analysis of 212 blood samples obtained from the portal vein for 5 days during laparotomy revealed a singular positive blood culture for *Staphylococcus aureus*.[38] Furthermore, no endotoxin was detected. The hypothesis of systemic passage through the lymph has been evaluated by preclinical experimental studies. In particular, ligation of the mesenteric lymphatic channel in a mouse model of AMI prevented the occurrence of MODS.[39]

The role of reperfusion syndrome after ischaemia in the systemic pathogenesis of AMI should be noted.[30] Reperfusion injury is greater than ischaemia itself, from the harmful effects of ROS created during reperfusion.[30] Although the role of AMI in the occurrence of a significant and deleterious systemic inflammatory response of the "sepsis-like" type must still be clarified, it is currently recommended to administer empiric antibiotic therapy in AMI.[40, 41] In a prospective observational study, Nuzzo et al showed that empiric, enteral antibiotic therapy (metronidazole 500 mg x 3/day combined with gentamicin 40 mg x 2/day) was independently associated with a reduction in the risk of intestinal necrosis.[42]

Diagnosis of acute mesenteric ischaemia

Clinical diagnostic criteria

Contrary to popular belief, most patients with AMI consult the emergency room at an early stage that is potentially reversible, but this condition is still insufficiently recognised. The majority of patients are initially present without a known cardiovascular disease history, without signs of acute abdomen, without organ failure and without elevation of plasma lactate.[43-45] On the other hand, acute abdominal pain is constant, apart from the particular case of intensive care patients receiving sedation.[1, 46] Abdominal pain is typically sudden, intense and resistant to non-opioid analgesics, continuous, peri-umbilical or diffuse, in contrast with abdominal palpation. It may be associated with vomiting (48 %), diarrhoea (31 %), digestive bleeding (18 %) which, being inconsistent and/or too late, has no validated diagnostic value.[43] Only one of these features of acute abdominal pain warrants the diagnostic suspicion of AMI and the performance of an urgent CT angiogram, including patients with a vascular history.[1, 47]

Radiological diagnostic criteria

The cornerstone of radiological diagnosis is the CT scan, which must be considered as a first-line examination.[48, 49] The scan should be performed as quickly as possible after the onset of symptoms and the radiologist should be informed of the diagnostic suspicion. The protocol should include acquiring images both before and after contrast injection at the arterial and venous portal stages. This allows for excellent visualisation of the vessels and facilitates a thorough analysis of the digestive tract. Additionally, the intrinsic contrast provided by intestinal fluid enhances the evaluation of the intestinal wall after contrast injection.[50] Therefore, it is not advisable to administer positive oral contrast to the patient. Due to the importance of early diagnosis, the injection must be performed for any suspicion of AMI, even in cases of degraded renal function, the risk of ignoring an AMI outweighs the risk of renal toxicity.[50, 51]

1. CT semiology

CT scan plays a dual diagnostic and prognostic role in patients with AMI. It allows the demon-

stration of vascular insufficiency and ischaemic intestinal lesions and eliminates other differential diagnoses.[51]

a) Vascular insufficiency

Intraluminal defects or mesenteric vessel occlusions demonstrate high diagnostic specificity (94-100 %), but their reported sensitivity is relatively low (12-15 %). These vascular anomalies are encountered in more than 75 % of patients.[52]

Occlusive forms. In occlusive forms of AMI, the scanner allows visualisation of the site of vascular obstruction, appearing as a filling defect of the vascular lumen. Demonstration of the occlusion is easier when it is proximal and reaches the large vessels. However, a distal vascular occlusion may be the only abnormality and should be looked for. Emboli usually originate from the heart or aorta. In most patients, blood flow is preserved in the proximal branches of the superior mesenteric artery (SMA) and the jejunal arteries. Acute embolic occlusion typically appears as a sharp interruption of the vessel. Smaller emboli may be located distally or only affect small branches. Associated infarctions of other organs (spleen, kidney, liver, lower limb) suggest an embolic mechanism.[53] Arterial thrombosis occurs mainly in the context of atherosclerotic disease and results from rupture of atherosclerotic plaque. Calcified or non-calcified plaques are frequently visible at the origin of the occluded vessel. SMA thrombosis is generally more proximal than emboli, visible in the first few centimetres of the artery. In most instances, a dissecting AMI is an extension of an aortic dissection. On CT, the dissection manifests as a linear intraluminal filling defect, representing the flap that separates the true and false lumens. Dissection may also arise in the context of large or medium vessel vasculitis. In such cases, CT imaging reveals a thickening of the vascular wall, possibly accompanied by perivascular fatty infiltration.

Non-occlusive forms. In non-occlusive mesenteric ischaemia (NOMI), the basic mechanisms are decreased flow and vasoconstriction. Therefore, a CT scan may show narrowed calibre veins, flattened *inferior vena cava*, diffuse irregularities or spasms of arterial branches and poor visualisation of intestinal arches and mural vessels.[54]

b) Ischaemic intestinal distress (PPP)

CT signs of intestinal distress are intestinal wall thickness, density and strengthening of the intestinal wall, fat infiltration and dilatation of affected loops-handle calibre.

Intestinal wall thickness. Intestinal wall thickening is a very common sign of intestinal ischaemia, usually due to early, reversible mural oedema or delayed, irreversible haemorrhage.[55] This sign has high sensitivity (85-88 %) but much lower specificity (61-72 %).[56] Thickening is much more pronounced in venous insufficiency. In cases of arterial obstruction, the intestinal wall usually has a thin appearance. This thinning is secondary to the loss of blood volume of the intramucosal arterial capillaries. It may be difficult to differentiate between a thinned wall and an absent wall enhancement. This sign has a high specificity (88 %) but a low sensitivity (40 %) for the diagnosis of AMI.[57]

Density and strengthening of the intestinal wall. Spontaneous increase in intestinal wall density has been well described in ischaemia associated with small bowel obstruction and also exists in patients suffering from AMI.[58] This hyperdensity is thought to result from submucosal or transmural haemorrhage. Evaluation of bowel wall enhancement plays a very important role in the diagnosis of AMI. Diminished or absent intestinal enhancement is a major sign. This feature has high specificity (88-100 %) and sensitivity ranging from 18 to 60 %.[59-62] The relatively low sensitivity is explained by the numerous anastomotic connections between the vessels that provide blood supply to the intestine. The definition of this sign is purely qualitative and it is best appreciated by comparing the affected segments to normal adjacent loops. Paradoxical hyper-enhancement of the intestinal wall can also be observed in AMI. In patients with hypovolemic shock, the small intestine appears dilated and typically shows increased and prolonged enhancement of the intestinal wall, thought to result from splanchnic vasoconstriction and slow perfusion, as this is usually observed in patients with NOMI.[63] A stratified enhancement, called a "target" appearance, can be observed. It is explained by the enhancement of the mucosa and the exter-

nal serosa surrounding a central oedematous layer of low density. The sign is observed in cases of arterial occlusion with reperfusion, but also in NOMI or venous ischaemia.[64]

Handle calibre. Dilatation of affected loops has a sensitivity of 39 to 67 % and a specificity of 29 % to 81 % in patients with AMI.[57, 59-62, 65] Dilatation is caused by reflex interruption of intestinal peristalsis or by irreversible transmural ischaemia causing exudation of fluid into the intestinal lumen. Dilatation was more frequently reported in patients with arterial occlusion than in those with venous occlusion or NOMI. One of the difficulties is not to confuse dilatation of the intestinal wall with ileus or mechanical obstruction.[66]

Fat infiltration. Mesenteric fat infiltration is one of the most sensitive signs of AMI (reported sensitivity up to 96 %). Therefore, it attracts attention and helps identify abnormal intestinal segments. However, its specificity is much lower and varies from 28 % to 68 %.[57, 59-62, 65, 67]

2. Signs of irreversible intestinal necrosis

In addition to making the diagnosis of AMI, radiologists must know how to differentiate ischaemia and intestinal necrosis, because the latter indicates a late form of AMI and leads to different management and prognosis. The radiologist must therefore estimate the probability of the presence of necrosis based on several signs. Intraperitoneal gas is the only pathognomonic sign of intestinal perforation and therefore of transmural necrosis.[54] Ideally, intestinal necrosis should be diagnosed and resected before this stage of perforation peritonitis. *Pneumatosis intestinalis* is also a sign suggestive of necrosis, but it is important to note that it can be seen in non-necrotic segments as well. Duron et al found that 47 % of AMI patients with parietal pneumatosis still had viable bowel, with only partial mural ischaemia without transmural necrosis.[68] Patients with gas in the splanchnic veins are more likely to have transmural necrosis than those with *pneumatosis intestinalis* alone.[69]

In AMI of arterial origin, intestinal dilatation higher than 25 mm and reduction or absence of enhancement of the digestive wall are more common in cases of intestinal necrosis. In a recent prospective study, only intestinal dilatation was retained as an independent factor of irreversible transmural necrosis, emphasising the importance of this sign which would reflect the interruption of intestinal peristalsis by damage to the deep muscular layers of the digestive wall. Thus, in the case of dilatation, the rate of surgical resection is significantly higher.[43]

Relevance of biomarkers

Improving the prognosis and treatment of AMI requires the identification of 1) sensitive and specific diagnostic biomarkers for the early and reversible stage of the condition and 2) new therapeutic avenues likely to limit reperfusion injury.[70] Today's conventional analytical approaches do not fulfil either of these objectives.[46] The complex histological structure of the intestinal wall, the metabolic modulation brought by dietary and bacterial environmental factors, the shared expression of proteins by the liver and the intestine and their hepatic metabolism through the portal blood are all reasons explaining the difficulties in identifying a molecular biomarker of diagnostic or therapeutic interest.[46, 71-73]

Certain blood laboratory abnormalities (hyperleukocytosis, metabolic acidosis, elevation of CRP, LDH, AST, CPK, alkaline phosphatase, phosphate, amylase) are inconsistently found during AMI, with low diagnostic performance.[61, 63] Pancreatic proteins, neurotensin, calcitonin, D-dimer and certain inflammatory mediators (IL-2, IL-6, TNF) are not sufficiently specific for intestinal epithelial damage. Gastrointestinal polypeptides (somatostatin, VIP, substance P), which are relatively specific, are rapidly eliminated by hepatic metabolism and are not accessible to peripheral blood testing.[74] The increase in hexosaminidase and L-lactate is too delayed.[43, 74] None of the markers with the best intestinal specificity today (intestinal fatty acid binding protein (IFABP), D-lactate, alpha-glutathione S transferase, ischaemia-modified albumin (IMA) and citrulline) has clinical application in the diagnosis of early AMI. [46, 71, 73, 75]

The positive or negative predictive value of L-lactate is not sufficient to make it a biomarker that can be used in the diagnosis of AMI. In non-occlusive AMI, the elevation of L-lactate may be linked solely to low circulatory flow. In occlusive AMI, Nuzzo et al have just shown that 49 % of patients presenting to the emergency room for an AMI do not have hyperlactataemia.[43]

Diagnosis of acute mesenteric ischaemia

Multimodal and multidisciplinary care

The AMI is a complex multistep process that begins with local reversible ischaemic attack of the intestinal mucosa, which may progress to irreversible transmural necrosis. It can be further complicated by MODS in its late form. Corcos et al formed the SURVI centre, providing emergency care for AMI whose focus is on preserving intestinal vitality through a multi-modal and multidisciplinary approach.

Results of their pilot study indicated that 2-year survival was 89 %, with a bowel resection rate of 39 % and long-term parenteral nutrition of 17 %. They also showed that so-called late forms of AMI have a much worse prognosis than early forms.[7]

Protocol for the treatment of acute mesenteric ischaemia

Protocol for the treatment of AMI aims to prevent possible organ failure and to initiate the early specific treatments related to the pathophysiology of AMI. Haemodynamic stabilisation is achieved by volume expansion with crystalloids and if necessary, with the administration of vasopressor amines. As the splanchnic circulation is particularly sensitive to vasoconstriction, it is crucial to navigate the resuscitation using precise cardiovascular monitoring. This approach helps prevent the administration of excessive doses of vasopressor amines in cases when blood volume is not optimised, as well as reduces the risk of exacerbating abdominal compartment

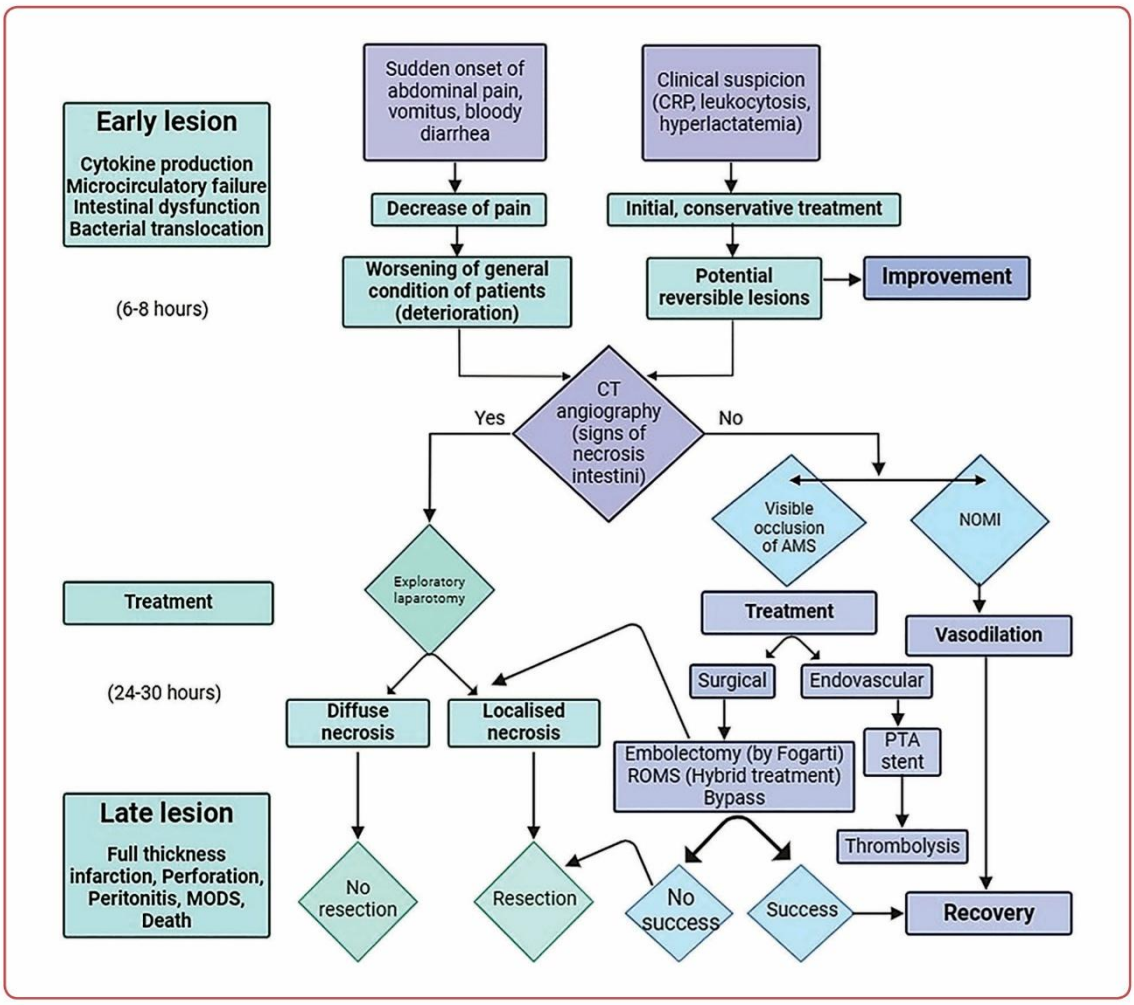

Figure 2: Algorithm for the treatment of acute mesenteric ischaemia (AMI)

MODS: multiple organ dysfunction syndrome; PTA: percutaneous transluminal angioplasty; AMS: superior mesenteric artery; NOMI: non-occlusive mesenteric ischaemia; ROMS: retrograde open mesenteric stent;

syndrome caused by unnecessary volume expansion.

Fasting and placing a nasogastric suction tube helps patients to relieve the reflex ileus. Before surgical treatment of occlusive AMI, it is necessary to quickly introduce a curative anticoagulant. For this purpose, the molecule of choice is unfractionated heparin (intravenously, 100 IJ/kg). The recommendation for antibiotic therapy is based on studies that have shown positive outcomes with selective digestive decontamination in intensive care, addressing potential infections from translocation or perforation of necrotic bowel tissue.[76]

The treatment protocol should combine broad-spectrum systemic intravenous antibiotic therapy (cefotaxime + metronidazole or piperacillin-tazobactam or cefepime + metronidazole) combined with antibiotic therapy administered enterally. The optimal method to administer the antibiotic therapy by the enteral route, particularly in patients with paralytic ileus or after gastrointestinal resection with stoma formation, still needs to be evaluated, especially in terms of duration of treatment.[76]

Indications for exploratory laparotomy

Exploratory laparotomy is necessary in several complicated conditions such as shock, suspected intestinal necrosis, peritonitis due to necrotic intestinal perforation or bacterial translocation and the necessity for open revascularisation (Figure 2). The predictive score for irreversible intestinal necrosis was established in order to more precisely identify the patients at high risk of irreversible necrosis, which requires urgent surgical evaluation.[43] This simple score includes three independent predictive factors, available after diagnosis of AMI: (1) presence of organ failure, (2) elevation of serum lactate > 2 mmol/L and (3) bowel dilatation > 25 mm on scan. Results from this study suggest that in the absence of these three factors, laparotomy can be avoided if percutaneous endovascular revascularisation is feasible.[43] With the slightest doubt, laparotomy should be widely used in other cases. Therefore, despite the null value in the positive diagnosis of AMI, L-lactate appears to have an important prognostic value, identifying the necrotic stage of AMI that requires laparotomy. As this study mainly included patients with occlusive AMI, these results cannot be generalised to non-occlusive AMI followed by systemic hypoperfusion condition of intensive care patients.[43]

Principles of digestive resection

In ideal clinical practice, revascularisation precedes and guides bowel resection, as this approach enhances the precision of defining the bowel resection margins. Revascularisation could significantly limit the extent of bowel resection. Bowel resection is predominantly conducted within peripheral healthcare centres lacking vascular surgery team while awaiting secondary transfer and in cases of pan-intestinal necrosis leading to short bowel as well as in cases of bowel perforation. The extent of digestive tract resection must be discussed collaboratively before proceeding with surgery (damage control surgery - DCS).[77] The determination of extent depends on the patient's factor: age, comorbidities and ability to consider life under total parenteral nutrition. In order to avoid dependence on total parenteral nutrition, it is recommended to maintain a minimum residual length of 100 cm of jejunum required for terminal jejunostomy, 65 cm of jejunum for jejunocolic anastomosis and 35 cm of jejunum for jejunoileal anastomosis, preserving ileocecal region.[6, 77] A postoperative surgical „second look" is not a standard procedure in every centre, but may be conducted in the presence of any suspicious remaining area or signs of secondary degradation.[78] Endoscopic control can be performed through stomas in the upstream and downstream segments of the intestine. Two practices that should be discouraged are resection - anastomosis and leaving segments of the small intestine closed in the abdomen while waiting for a „second look."

Revascularisation strategies

Mesentery revascularisation techniques depend upon the aetiology of arterial occlusion - whether atherothrombosis or embolism, along with the morphological appearance of arterial lesions and the indication for laparotomy in case of suspected bowel necrosis. The target artery that should be revascularised is the SMA. Most studies regarding this issue are single-centre and retrospective. A retrospective comparison of endovascular and open surgical revascularisation is often biased and may lack significance because these techniques are not applied to the same patients. The current problem appears to be more related to mesenteric revascularisation in AMI, which is still insufficient. This was shown in US study involving 23,744 patients with AMI, among whom only 3 % benefited from revascularisation.[79] However, approximately 70 % of patients with acute SMA obstruction require revascular-

isation to survive, while the remaining 30 % can be saved by bowel resection alone.[78, 79]

Whenever possible, endovascular revascularisation techniques are preferred, as they offer significant benefits in terms of postoperative morbidity and mortality, length of hospitalisation and nutritional recovery. Two publications from the Swedvasc registry report historical results of SMA revascularisation for AMI for the periods between 1987 and 1998 as well as between 1999 and 2006.[80, 81] Swedvasc is a vascular registry established in 1987, which collects information about more than 90 % of vascular surgical procedures performed in Sweden. Overall, surgical revascularisation quadrupled from 1999 to 2006, while the number of endovascular revascularisations increased sixfold. Although total mortality caused by this condition decreased, the reduction was observed only in patients treated with an endovascular strategy. With a similar length of bowel resection in both revascularisation techniques, the endovascular strategy stood out as an independent survival factor, as revealed by the multivariate analysis. However, the main reason for the success of the endovascular strategy was the availability of surgical revascularisation as an option in case of failure. While the data from the Swedvasc register for the period 2009-2015 are not currently available, it appears that since 2009, a significant proportion of the procedures for the AMI may have been endovascular.[81] Similar observations emerge from an analysis of the National Inpatient Sample (NIS) database in the United States. The NIS is a high-quality database that contains information for 20 % of hospitalisation episodes from nearly 1,000 US hospitals. Among 679 patients treated for AMI between 2005 and 2009, 514 (76 %) underwent open, surgical revascularisation, while 165 (24 %) patients were treated with an endovascular procedure.[78, 80] The proportion of patients undergoing endovascular intervention increased from 12 % in 2005 to 30 % in 2009. Mortality was 39 % after surgery and only 25 % after endovascular procedures. Among survivors, the proportion of patients requiring total parenteral nutrition was significantly higher after surgery than after an endovascular strategy.[78, 80]

In their early stages, AMIs of embolic origin are most often available for endovascular revascularisation through techniques such as thromboaspiration or fibrinolysis *in situ*. The technique of choice in their later stages is open, surgical embolectomy of SMA. The technique involves approaching the SMA at the root of the mesentery and dissecting the truncal SMA. Longitudinal arteriotomy is conducted to allow embolectomy with different Fogarty probes of the proximal SMA, different collateral arteries and distal dividing branches. The artery is then closed by suturing a patch (graft), whether biological or prosthetic, to prevent stenosis. An additional injection of urokinase can be applied *in situ* by direct puncture of the SMA, without increasing the risk of bleeding.[81] In atherothrombotic origin AMI, when open surgical revascularisation is chosen, two applicable techniques are retrograde open mesenteric stent (ROMS) and bypass (Figure 2).

In recent years, ROMS has become the hybrid surgical technique of choice in atherothrombotic AMI requiring exploratory laparotomy. It combines open surgical techniques with endovascular techniques. ROMS is performed by laparotomy, with an approach to the SMA similar to that used for embolectomy. Direct retrograde puncture of the SMA enables retrograde catheterisation of the SMA towards the aorta, as close as possible to the lesion. This technique has several advantages as it allows exploration of the small intestine by laparotomy. Compared to bypass, it enables faster vascular repair with comparable patency rates, while avoiding aortic clamping as well as coverage problems and infection. The most commonly used stents are covered steel balloon-mounted stents. This approach allows the combination of thrombectomy or endarterectomy procedures, as SMA atheromatous occlusions may be associated with thrombosis *in situ* or multiple distal occlusive lesions. In case of catheterisation failure, bypass surgery is always possible.[80, 81]

Mesenteric bypasses are considered as the last choice of revascularisation techniques for SMA. Even with the decreasing frequency of their use in AMI due to the advent of ROMS and endovascular techniques, bypasses continue to be indicated. They are recommended in cases of SMA stent thrombosis, ROMS failure, bypass thrombosis and for long and complex occlusions whose morphology is not suitable for endovascular treatment. Many centres prefer retrograde ilio-mesenteric bypasses as they do not require aortic clamping. The most commonly used graft is a ring-shaped Gore-Tex prosthesis. Bypasses should be isolated from alimentary loops to minimise the risk of prosthesis infection and secondary alimentary fistula.[81]

isation to survive, while the remaining 30 % can be saved by bowel resection alone.[78, 79]

Whenever possible, endovascular revascularisation techniques are preferred, as they offer significant benefits in terms of postoperative morbidity and mortality, length of hospitalisation and nutritional recovery. Two publications from the Swedvasc registry report historical results of SMA revascularisation for AMI for the periods between 1987 and 1998 as well as between 1999 and 2006.[80, 81] Swedvasc is a vascular registry established in 1987, which collects information about more than 90 % of vascular surgical procedures performed in Sweden. Overall, surgical revascularisation quadrupled from 1999 to 2006, while the number of endovascular revascularisations increased sixfold. Although total mortality caused by this condition decreased, the reduction was observed only in patients treated with an endovascular strategy. With a similar length of bowel resection in both revascularisation techniques, the endovascular strategy stood out as an independent survival factor, as revealed by the multivariate analysis. However, the main reason for the success of the endovascular strategy was the availability of surgical revascularisation as an option in case of failure. While the data from the Swedvasc register for the period 2009-2015 are not currently available, it appears that since 2009, a significant proportion of the procedures for the AMI may have been endovascular.[81] Similar observations emerge from an analysis of the National Inpatient Sample (NIS) database in the United States. The NIS is a high-quality database that contains information for 20 % of hospitalisation episodes from nearly 1,000 US hospitals. Among 679 patients treated for AMI between 2005 and 2009, 514 (76 %) underwent open, surgical revascularisation, while 165 (24 %) patients were treated with an endovascular procedure.[78, 80] The proportion of patients undergoing endovascular intervention increased from 12 % in 2005 to 30 % in 2009. Mortality was 39 % after surgery and only 25 % after endovascular procedures. Among survivors, the proportion of patients requiring total parenteral nutrition was significantly higher after surgery than after an endovascular strategy.[78, 80]

In their early stages, AMIs of embolic origin are most often available for endovascular revascularisation through techniques such as thromboaspiration or fibrinolysis *in situ*. The technique of choice in their later stages is open, surgical embolectomy of SMA. The technique involves approaching the SMA at the root of the mesentery and dissecting the truncal SMA. Longitudinal arteriotomy is conducted to allow embolectomy with different Fogarty probes of the proximal SMA, different collateral arteries and distal dividing branches. The artery is then closed by suturing a patch (graft), whether biological or prosthetic, to prevent stenosis. An additional injection of urokinase can be applied *in situ* by direct puncture of the SMA, without increasing the risk of bleeding.[81] In atherothrombotic origin AMI, when open surgical revascularisation is chosen, two applicable techniques are retrograde open mesenteric stent (ROMS) and bypass (Figure 2).

In recent years, ROMS has become the hybrid surgical technique of choice in atherothrombotic AMI requiring exploratory laparotomy. It combines open surgical techniques with endovascular techniques. ROMS is performed by laparotomy, with an approach to the SMA similar to that used for embolectomy. Direct retrograde puncture of the SMA enables retrograde catheterisation of the SMA towards the aorta, as close as possible to the lesion. This technique has several advantages as it allows exploration of the small intestine by laparotomy. Compared to bypass, it enables faster vascular repair with comparable patency rates, while avoiding aortic clamping as well as coverage problems and infection. The most commonly used stents are covered steel balloon-mounted stents. This approach allows the combination of thrombectomy or endarterectomy procedures, as SMA atheromatous occlusions may be associated with thrombosis *in situ* or multiple distal occlusive lesions. In case of catheterisation failure, bypass surgery is always possible.[80, 81]

Mesenteric bypasses are considered as the last choice of revascularisation techniques for SMA. Even with the decreasing frequency of their use in AMI due to the advent of ROMS and endovascular techniques, bypasses continue to be indicated. They are recommended in cases of SMA stent thrombosis, ROMS failure, bypass thrombosis and for long and complex occlusions whose morphology is not suitable for endovascular treatment. Many centres prefer retrograde ilio-mesenteric bypasses as they do not require aortic clamping. The most commonly used graft is a ring-shaped Gore-Tex prosthesis. Bypasses should be isolated from alimentary loops to minimise the risk of prosthesis infection and secondary alimentary fistula.[81]

A special case of acute non-occlusive mesenteric ischaemia

Treatment of non-occlusive AMI primarily focuses on addressing the underlying cause of the shock state responsible for the low mesenteric flow. To suppress vasospasm, an option is the treatment of intra-arterial vasodilation of the SMA using substances such as papaverine or ilomedin, facilitated by the placement of an *in situ* catheter.[82, 83]

Conclusion

Predictive factors of mortality in AMI include old age, hyperlactataemia, metabolic acidosis, hypoxaemia, pneumatosis of the alimentary wall, MODS and sepsis. An active approach to diagnosis and treatment of AMI is crucial for reducing the time of recurrence and improving the prognosis, which, despite efforts, remains associated with high mortality rate. In summary, the morbidity and mortality rates remain high in cases of AMI. Over the last two decades, subtle improvements in survival have been achieved, probably due to the more liberal utilisation of second-look laparotomy, even in the older patient population. Embolic aetiology, signs of intestinal infarction at initial presentation and the presence of systemic atherosclerosis are predictors of poor outcome. The proportion of endovascular revascularisations has increased in the last decade and holds promise for further improvements facilitated by potential technical advances, including a broader range of low-profile and quick-change devices, as well as embolisation protection devices. Careful patient selection, procedural planning, meticulous technique and liberal use of the hybrid technique with retrograde access according to guidelines of World Society of Emergency Surgery are expected to additionally improve the outcome of endovascular treatment of AMI. Enhancement in overall outcome will largely depend on prompt diagnosis and appropriate therapy, whether through open or endovascular approaches, along with early and repeated bowel assessment.

Ethics

This study was a secondary analysis based on the currently existing data bases including *PubMed* and did not directly involve with human participants or experimental animals. The ethics approval was not required for this paper.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

Author ORCID numbers

Zoran Matković (ZM):
0000-0002-5216-5793
Uglješa Maličević (UM):
0009-0004-7649-186X
Milica Gajić Bojić (MGB):
0009-0006-8433-7808
Aleksandra Krivokuća (AK):
0009-0004-9431-3848
Đorđe Đukanović (ĐĐ):
0000-0002-9887-9695

Nataša Đekić Matković (NĐM):
0009-0005-9731-6320
Zoran Aleksić (ZA):
0009-0002-7047-3500

Author contributions

Conceptualisation: ZM, UM, ZA
Methodology: ZM, NĐM, ĐĐ
Formal analysis: UM, ĐĐ, MGB, AK
Investigation: ZM, ZA, UM
Writing original draft: ZM, ZA
Writing – review and editing: UM, MGB, AK, ĐĐ, ZM
Validation: ZM, ZA, UM, MGB
Visualisation: ZM, MGB, AK
Supervision: ZM, ZA, UM, MGB, AK, ĐĐ
Project administration: ZM, NĐM

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Medical, Surgical and Experimental Approaches to Acute Mesenteric Ischemia and Reperfusion

Zoran Matkovic[1], Zoran Aleksic[2,3]

[1]Department of Abdominal Surgery, General Hospital Doboj. Doboj, Bosnia and Herzegovina

[2]Clinic for General and Abdominal surgery, University Clinical Centre of the Republic of Srpska, BanjaLuka. Banja Luka, Bosnia and Herzegovina

[3]Faculty of Medicine, University of Banja Luka. Banja Luka, Bosnia and Herzegovina,

Coresponding autor
Zoran Matkovic, MD. Department of Abdominal Surgery, General Hospital Doboj. Doboj, Bosnia and Herzegovina. Phone: :+387 65 740 459. E-mail : zoranmatna@gmail.com.

ABSTRACT:
Background: Acute mesenteric ishemia(AMI) is a rare but very serious disease with high rate of mortality and morbidity. About 1-2% of all gastrointestinal disease is AMI. Mortality is about 60-80% and depends of time between starting of symptoms and establishing of diagnosis, type AMI, comorbidities. AMI is often in older population with coronary syndrom and atrial fibrilation. AMI may be occlusive(embolisatio arteriae mesentericae superior(AMS), or thrombosis of AMS, mesenterial vein thrombosis) and nonoclusive form(NOMI). NOMI is rising in critical ill patients in shock or sepsis. Pathophysiology of AMI is very complex and significant role in this proces has ischemia and also reperfusion. Reperfusion injury including oxidative stres, inflamation, infection. The best diagnostic approach is CT angiography but after high clinical suspicion on AMI. Patients have sudden, catastrophic abdominal pain, vomitus, bloody diarrhoea. Therapy is multidisciplinary-basic treatment(resuscitation with cristaloids, antibiotic, anticoagulans...), surgical treatment-resection necrotic segments of intestinum without anastomosis or endovascular treatment. In early phases conservative treatment is possible(vasodilatation, thrombolysis). In some countries there are Intestinal Stroke Centers (ISC) in which patients with AMI have better prognosis. Because of progressive nature of AMI(rapide worsening) rare are clinical study,but there are many experimental study on animal models. Most of experimental study investigate protective effects of some supstances on damage on intestinum and remote organs during ishemia and reperfusion**. Objective:** To present literature data of clinical and experimental study, describe experiments on animal models and mention supstances whit promising results in protective strategies during AMI. **Methods:** We analysed Pubmed by using mesh terms such as acute mesenteric ischemia, intestinal injury, reperfusion, experimental study, clinical and therapeutic approach**. Results**: Sudden abdominal pain resists on **o**pioids analgetics**,** high rate of CRP, hyperlactatemia, increase of D dimer is enough for suspicion of AMI. Often is delayed in establishing of diagnosis of AMI. CT angiography has sensitivity of 94%. Pneumatosis is sign of necrosis of intestinal wall. Classical surgical approach is dominant, more than 70%,. Endovascular treatment became often last few years. Experimental studies investigate occlusion of AMS with atraumatic clamp, with schemia and reperfusion in different intervals Most animals models are on wistar male rats. **Conclusion:** AMI has still high rate of mortality. Better diagnostic and therapeutic principles (shorter interval between appearance of symptoms and starting of therapy, multidisciplinary approach, higher percent of endovascular procedures), could decrease mortality. Experimental studies on animal models may be succesfull in development of new clinical, conservative approaches in the early phases of AMI in the future.
Keywords: acute mesenteric ischemia, reperfusion, experimental study.

1. BACKGROUND

Acute mesenteric ishemia (AMI) is a rare but very serious urgent disease with high mortality and morbidity. AMI accounts for 1 do 2% of all gastrointestinal diseases. It is thought that 1 in 100 patients with a clinical picture of acute abdomen actually has AMI. Mortality is between 60 and 80% (1). AMI is a critical and sudden interruption of blood

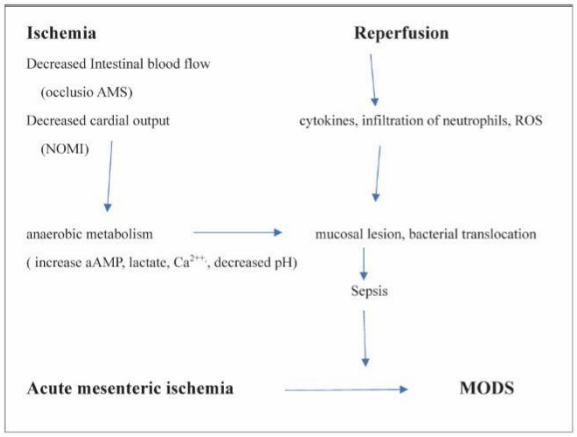

Figure 1. Patophysiologic mechanisms during AMI

flow to the mesentery with funkctional and structural changes in the intestine and distant organs (2). This complex disease most often affects the elderly population with advanced atherosclerosis,with atrial fibrilation, heart failure and other comorbidities.In people over 80 years of age , AMI is a more common cause of acute abdomen than acute appendicitis (3, 4). AMI can be divided into occlusive and non-occlusive forms.(NOMI)

The occlusive form includes:

* Embolization of superior mesenteric artery(AMS) in 40-50% cases.

* Thrombosis of AMS in 25-30% cases.

* Mesenteric venous thrombosis (MVT)in 10-18% cases.(in malignancy, necrotizing pancreatitis, myeloproliferative diseases, protein C and S deficiency, intraabdominal infection,venous trauma).

The non-oclusive form accounts for 20% of all cases of AMI and is found in critically ill patients and in a state of shock, sepsis, where there is hypoperfusion due to the centralization of blood flow caused by the use of vasoconstrictors (5, 6). Despite advances in diagnosis and therapy, AMI remains a highly lethal disease for more than 50 years with perioperative mortality of over 50%. Overal survival has not changed for decades (7). One of the main problems is the delay in establishing a diagnosis and starting treatment. The presentation of the disease is catastrophic pain, the intensity of which does not corespond to the palpation findings. The small intestine is very sensitive to ischemia. The intestine requires about 25% of the minute volume and the pain occurs due to the disproportion between the needs and the amount of available oxygen (8). In anaerobic conditions, pyruvate turns into lactate, which irritates the nerves in the intestines (5, 9, 10). After 6 to 8 hours, there is a reduction in pain due to nerve damage, there is an asymptomatic interval that is temporary and marks the begining of the disaster (11). With the duration of the interruption of circulation, there is a violation of the intestinal barrier and the occurence of bacterial translocation. Endotoxins and bacteria cross the mesenteric lymph nodes and portal vein and s systemic disorder occurs. Necrosis of the intestinal wall occurs after ischemia (11). Initial clinical suspicion that is AMI is very important (12).

The goal is to shorten the time from the onset of symptoms and diagnosis to therapeutic treatment. Every 24-hour delay doubles mortality (2). Survival is about 50% when the diagnosis is made in the first 24 hours, but depends a lot on the AMI subtype. For example: improvement of perfusion and vasospasm in NOMI will enable a favorable outcome in a significant percentage of patients. MVT is considered to have a very poor prognosis. The establishment of blood flow leads to reperfusion, which often causes more damage than that caused by the previous ischemia. The shock wave of oxygen leads to the oxygen paradox (Dual-Hit hypothesis) (13). Bad effects on the organs can be reduced by the action of antioxidants in that phase (14). In the literature this is known as an

Figure 2. Gangrene of intestinum

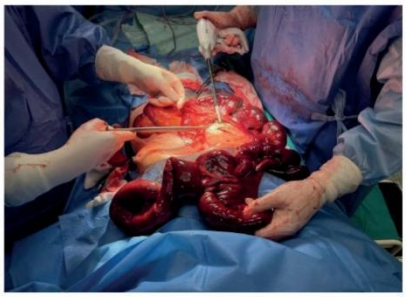

Figure 3. Resection of gangrenous segment of intestinum

intestinal ischemia-reperfusion lesion.(IIRI).

In 1986, Parks and Granger were the first to describe the harmful effect of reactive oxygen species created during reperfusion, noting that reperfusion injury is greater than ischemia itself (15). What sets this ischemic reperusion injury apart from the same lesions on other organs(myocardial infarction, cerebrovascular insult, liver and kidney lesions) is the certain occurence of a septic condition due to the already mentioned disruption of the intestinal barrier and bacterial translocation. Multiorgan dysfunction syndrome (MODS) caused by combination of AMI and sepsis is the most common cause of death (16). The mechanism of changes includes oxidative stress, inflammation, the action of the microbiome (17, 18).

Acute mesenteric ischemia MODS

With the interruption of circulation, anaerobic metabolism occurs. The level of adenosine triphosphate decreases, acidity increases, disturbances in the operation of ion pumps occur, which leads to the activation of phospholipase A2 and lipid peroxidation by opening the mitochondrial transition permeable pore (mPTP). Establishment of blood flow leads to the appearance of reactive oxygen species (ROS). These highly reactive substances can directly damage tissue by acting on the level of mitochondria, but they also affect the infiltration of neutrophils through interleukins and adhesive molecules and in the inflamatory reaction, also leading to cell death (15, 16, 18).

Clinical signs and symptoms	abdominal pain(100%) vomitus(48%) blady diarrhoea (31%)
Laboratory parameters	CRP(100 and more), leukocytosis, increase value of D-dimer, hyperlactatemia (2 mmol and more)
New serological biomarkers of AMI	IMA(ischemic modified albumin),I-FABP(Intestinal faty acid protein), D-lactat, L citrulin.
CT angiography (sensitivity 94%), CT scan	aperistalsis, dilation of intestinum, pneumatosis of intestinal wall (19).

Table 1. Diagnostic findings of AMI

Basic(initial) treatment	iv resuscitation,NG tube,urinary cateter, intravenous antibiotics,anticoagulans,opioids analgetics
Classical surgical treatment (about 80%)	exploratory laparotomy, resection of necrotic segments of intestinum without anastomosis.
Endovascular treatment (about 20%)	Embolectomy, thrombolysis, stenting,by pass
Hybrid treatment	Simultaneous work of abdominal surgeon and vascular surgeon ROMS(retrograde open mesenteric stent)

Table 2. Therapeutic approach

Animals models	Male rats (more than 85%of all study), mice, cats, pigs
Type of ischemia	Occlusion of AMS, occlusion AMS and VMS, low flow occlusion, Ligature of AMS (rare), Porcine model (percutaneous or endovascular Embolization of AMS, Murine mode l(segmental vascular occlusion)
Duration of ischemia	30 -90 minuts
Duration of reperfusion	90-120 minuts.

Table 3. Experimental approach. Occlusion of AMS is usually with atraumatic clamp in most studies(25).

Antioxidants	SOD(superoxide dismutase), GSH(glutation), alfa tokoferol, melatonin ,minocycline,curcumin,cysteine, candesartan...
Antiinflammatory and antiapoptotic substances	Ghrelin, dexmedetomidin, levosimendan, flurbiprofen axetil,,methylprednisolone...
Other substances	sevoflurane, sesamin, iloprost, astaxanthin, magniferin, melatonin,sulphoraphane, papaverine, albumin, albendazole,pentoxifilin, nitrat vasodilatators, low molecular weight heparin, nitroindazol, alopurinol, simvastatin, diltiazem, dioscin, ethanol, estradiol, agmantin,glutamin...

Table 4. List of substances with protective effects during AMI.

2. OBJECTIVE

The aim of this article was to present actual literal data of clinical (patophysiology, diagnostic and therapeutic approach of AMI) and experimental study on animals which investigate AMI. Also, we want to mentioned pharmacological supstances applied in experimental study with protecting effects during ishemia and reperfusion injury of mesenterium.

3. MATERIAL AND METHODS

We analysed recent articles on Pubmed ussed mesh terms such as acute mesenteric ischemia, intestinal injury, reperfusion, experimental study, animals models, clinical and therapeutic approach, and remote organs injury.

4. RESULTS

Numerous pharmacological supstances have protective effects on inestinum and remote organs during acute mesenteric ischemia in animal models (30).

5. DISCUSSION

CT angiography is the best diagnostic test for AMI (sensitivity about 94%, specificy 95%) (19). On CT scan we can see absence of peristalsis, thickening and pneumatosis of bowel wall. Pneumatosis is a sure sign of necrosis. Laparotomy is consider as a gold standard, but it si often late. Laboratory parameters are not specific, but can indicate on development of an acute abdominal surgical disease(leukocitosis, increase of CRP, D- dimer, lactate). New serological biomarkers such as ischemia modified albumin (IMA), intestinal faty acid binding protein (I-FABP), D lactate i L citrulin are not yet used in routine practice (Table 1) (20).

Therapeutic principle is mutidisciplinary approach-cooperation of general or abdominal surgeon, vascular surgeon, interventional radiologist, intensivist, according to the recommendations of WSES (World Society of Emergency Surgery). Corcos was initiated the establishment Intestinal Stroke Center in France, 2016 year. Later publications proved improved survival of patients with AMI treated at such centers (22). Generous rehydration is required (1-2 ml ringer lactate/kg/h), due to losses in the intestinal lumen and peritoneal cavity. Correction of metabolic acidosis and hypokalemia is also required, then placement of nasogastric tube, urinar catheter to measure hourly diuresis. Intravenous antibiotics and anticoagulans (bolus of heparin) and finally surgical treatment are applied. Surgical resections are performed without anastomosis, (Damage control surgery) or if ischemic changes are observed, revascularization is performed. A hybdrid treatment is also possible in larger centers- simultaneos work of abdominal and vascular surgeon (Table 2). An alternative to the surgical approach before the occurence of irreversible changes are anticoagulans, thrombolitics, vasoactive drugs.

According to Fuglseth, timely anticoagulant treatment enables recanalization in 80% of cases (6). Understanding of pathophysiological processes and cascade that occurs during ischemia and reperfusion can conntribute to the development of new therapeutic strategies. Due to the nature of the disease, (rapid deterioration in a short time interval) is not possible for ethicals reasons to conduct clinical studies. That is why experimental animal models were developed. Research therapy of AMI involves a pharmacological approach, and in the last ten years, many substances have appeared with some promising results (23). There is also a non pharmacological approach such as pre- and post-condtitioning. Repeated short-term ischemia and reperfusion make the target intestinal tissue and distant organs more resistant to ischemia-reperfusion injury (24).

Experimental animal models

Most of the experiments were conducted on male Wistar rats. The age of the animals was from 6 to 18 weeks, the weight of the animals was from 200 to 350 grams (25, 26). The most common and best described is the superior mesenteric artery occlusion model. The expected period of ischemia is followed by release of the clamp and a period of reperfusion. Reperfusion is recognized by the appearance of pulsations, the establishment of peristalsis and the light-red color of the intestines (26). Microvascular, atraumatic clamps are used for occlusion to avoid endothelial lesions and to adequately evaluate subsequent reperfusion. The duration of occlusion is between 30 and 90 minutes. Reperfusion time is up to 120 minutes. Taking into account the metabolic rate of rats, this corresponds to clinical events in the human organism, where it takes 6 to 8 hours to reach complete ischemia and reperfusion (27). Experiments with complete vascular occlusion were also performed. A porcine model-percutaneous or endovacular embolization is also used. This model is the closest to the clinical event during acute mesenteric ischemia-the flow occlusion is done internally(from the lumen) as an embolus or thrombus does (25, 26, 28). Described works with segmental vascular occlusion (clamping of individual branches) are known as the Murine model (29). The most frequently measured parameters of oxidative stress (SOD, catalase (CAT), glutation (GSH), hydrogen peroxide, superoxide anion radical), markers of inflammation (TNF-alfa, IL1, IL 6)and endothelial dysfunction (Nitric oxide (NO), Intercellular adhaesion molecule-1, (ICAM-1), Vascular Cell adhaesion molecule, (VCAM-1)). Liver enzymes (aspartat aminotranferase (AST), alanin aminotransferase (ALT), TBARS, (thiobarbituric acid reactive substances) were examined (30). Tissue samples (ileum, liver, kidneys, lungs and heart) are used for assesment of apoptosis.

Aplication of substances in experimental studies were as pretreatment, (most common) in the phase of ischemia (rare) and in the start of reperfusion (30). It is considered that lesions of remote organs mostly arises during early phases of reperfusion, about 1 to 6 hours by signaling way Nuclear factor kapa beta (NF-kB). Most often is a lesion of liver because of blood flow from mesenterium through vena porte (31). Regarding patophysiology of AMI it is develope different mechanisms of protection (32). Numerous substances are ussed in experimental conditions, some of them have shown excellent results and were well tolerated. These agents can act on next different mechanisms: antioxidative, antiinflammatory, antiapoptotic, reinforcement of the cell membrane, amplification of energetic resource of the cell (impact on mitochondria) (Table 4) (14, 27, 30-36). List of substances is not final because the effects of other substances are examined on ischemia-reperfusion lesion of mesenterium and remote organ lesions.

6. CONCLUSION

Acute mesenteric ischemia still remain a clinical chalenge because late recognition and delayed therapeutic treatment are very often. Outcome of treatment actualy depends from interval between starting of symptoms and starting of therapy. Surgical approach (laparotomy, resection) have still a dominant role in the treatment, but there are more often endovascular treatment in the last years. In the last decades there are more and more conservative-pharmacological approach in situations where intestinum is still vital. This approacht arrising defence from reperfusion injury and remote organ injury after recovery of circulation. Most of study is experimental and relate on pretreatment by pharmacological agents. In the future we expect wider aplication of conservative treatment on patients with AMI.

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