Uniwersytet Przyrodniczy we Wrocławiu
Wydział Medycyny Weterynaryjnej

PRACA DOKTORSKA

Doctoral thesis

Ocena kardioprotekcyjnego działania dowieńcowo podawanego kwasu acetylosalicylowego na świńskim modelu niedokrwienia/reperfuzji – badania in vivo.

Assessment of the cardioprotective effect of intracoronary acetylsalicylic acid in a porcine model of ischemia/reperfusion - in vivo study.

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SPIS TREŚCI

WYKAZ UŻYTYCH SKRÓTÓW

ABTS	sól diamonowa 2,2’-azobis(3-etylobenzotiazolino-6-sulfonianu)
ASA	kwas acetylosalicylowy, aspiryna
ATP	adenozyno-5′-trifosforan
COX	cyklooksygenaza
DNA	kwas deoksyrybonukleinowy
EKG	badanie elektrokardiograficzne
GPx	peroksydaza glutationowa (ang. *glutathione peroxidase*)
GR	reduktaza glutationowa (ang. *glutathione reductase*)
GSH	forma zredukowana glutationu
GSSG	forma utleniona glutationu
GST	S-transferaza glutationowa (ang. *glutathione S-transferase*)
LAD	gałąź przednia zstępująca lewej tętnicy wieńcowej
LCX	gałąź okalająca lewej tętnicy wieńcowej
LDL	lipoproteiny o małej gęstości (ang. *low-density lipoprotein*)
LF	lipofuscyna (ang. *lipofuscin*)
MDA	dialdehyd malonowy (ang. *malondialdehyde*)
MI	zawał mięśnia sercowego (ang. *myocardial infarction*)
NADPH	zredukowana forma fosforanu dinukleotydu nikotynoamidoadeninowego
OSI	wskaźnik stresu oksydacyjnego (ang. *oxidative stress index*)
PCI	przezskórne interwencje wieńcowe (ang. *percutaneous coronary interventions*)
ROS	reaktywne formy tlenu (ang. *reactive oxygen species*)
TAC	całkowita zdolność antyoksydacyjna (ang. *total antioxidant capacity*)
TOS	całkowity status oksydacyjny (ang. *total oxidative status*)
VF	migotanie komór (ang. *ventricular fibrillation*)
VT	tachykardia komorowa (ang. *ventricular tachycardia*)

STRESZCZENIE

Niedokrwienie i zawał mięśnia sercowego oraz inne choroby układu krążenia są wymieniane wśród głównych przyczyn zgonów ludzi w krajach uprzemysłowionych. Zawał mięśnia sercowego, wraz z chorobą niedokrwienną serca, stanowią najczęstszą przyczynę niewydolności serca, czyli stanu, w którym serce nie jest w stanie dostarczyć odpowiedniej ilości krwi do narządów organizmu. Wśród najistotniejszych powikłań niedokrwienia i zawału mięśnia sercowego wskazuje się rozwój zaburzeń rytmu serca, zwłaszcza zagrażających życiu arytmii komorowych: częstoskurczu komorowego i migotania komór.

Patofizjologia niedokrwienia mięśnia sercowego jest złożona, a w medycynie człowieka jako jego główną przyczynę wskazuje się miażdżycę i powstające w jej przebiegu blaszki miażdżycowe, które po oderwaniu mogą doprowadzić do zamknięcia światła naczyń wieńcowych, skutkującego niedokrwieniem mięśnia sercowego. Najczęstszym miejscem okluzji naczyń zaopatrujących serce jest gałąź przednia zstępująca lewej tętnicy wieńcowej. Przywrócenie krążenia w niedokrwionym obszarze mięśnia sercowego może jednak skutkować dalszym uszkodzeniem mięśnia sercowego określanym jako uszkodzenie niedokrwienno-reperfuzyjne. Jego patogeneza jest złożona, a wśród potencjalnych procesów zaangażowanych w rozwój uszkodzenia niedokrwienno-reperfuzyjnego wymienia się aktywację i agregację płytek krwi razem z następstwami tych zjawisk oraz zwiększoną produkcję reaktywnych form tlenu, która dodatkowo prowadzi do nasilenia zjawiska zwanego stresem oksydacyjnym.

Mianem stresu oksydacyjnego określamy zaburzenie równowagi pomiędzy produkcją reaktywnych form tlenu a działaniem mechanizmów antyoksydacyjnych organizmu. Reaktywne formy tlenu mogą powstawać zarówno ze źródeł endogennych, jak i egzogennych, a ich nadmierna produkcja i nagromadzenie prowadzi do uszkodzenia komórek, tkanek, białek, lipidów i DNA. Do najczęstszych reaktywnych form tlenu zaliczane są anionorodnik ponadtlenkowy, nadtlenek wodoru, rodnik hydroksylowy oraz tlen singletowy. Do ochrony ustroju przed reaktywnymi formami tlenu służą nieenzymatyczne i enzymatyczne mechanizmy antyoksydacyjne. Stres oksydacyjny stanowi jedną z przyczyn chorób sercowo-naczyniowych, a jego rola została potwierdzona w patogenezie i progresji m.in. miażdżycy, niedokrwienia i niewydolności serca. Do oceny nasilenia stresu oksydacyjnego wykorzystywane są enzymatyczne i nieenzymatyczne markery stresu oksydacyjnego.

Jednym z leków o działaniu przeciwpłytkowym wykorzystywanych w prewencji i leczeniu niedokrwienia i zawału mięśnia sercowego jest kwas acetylosalicylowy, potocznie zwany aspiryną. Oprócz hamowania aktywacji płytek krwi wykazuje on działanie przeciwzapalne, przeciwbólowe i przeciwgorączkowe. Jako główny mechanizm działania kardioprotekcyjnego kwasu acetylosalicylowego wskazuje się jego działanie antyagregacyjne w stosunku do płytek krwi, jednak sugerowany jest również antyoksydacyjny potencjał aspiryny. Kwas acetylosalicylowy u ludzi z niedokrwieniem i zawałem mięśnia sercowego podawany jest doustnie lub dożylnie, a nową koncepcją jest jego dowieńcowe podanie podczas epizodu ostrego niedokrwienia mięśnia sercowego w celu zmniejszenie uszkodzenia poreperfuzyjnego mięśnia sercowego. Możliwość wykorzystania dowieńcowej drogi podania w celu efektywniejszego działania kwasu acetylosalicylowego w niedokrwieniu mięśnia sercowego znajduje swoje potwierdzenie w literaturze, gdzie wskazuje się na większą skuteczność dowieńcowego niż dożylnego podania innych substancji o działaniu przeciwpłytkowym.

W badaniach naukowych dotyczących patofizjologii, diagnostyki czy leczenia niedokrwienia i zawału mięśnia sercowego wykorzystuje się zwierzęce modele chorób serca, zwłaszcza świński model niedokrwienia i zawału mięśnia sercowego. Świnia, ze względu na podobne do ludzkich anatomię serca, krążenia wieńcowego czy mechanizmów arytmogennych stanowi dobry model niedokrwienia mięśnia sercowego u ludzi. Zazwyczaj model ten jest osiągany poprzez przeznaczyniową okluzję naczyń wieńcowych, m.in. z wykorzystaniem balonów angioplastycznych. Najczęściej zamykanym naczyniem jest gałąź przednia zstępująca lewej tętnicy wieńcowej, jednak rzadko przeprowadza się okluzję jej proksymalnego odcinka, co związane jest z wysoką śmiertelnością zwierząt podczas takiej procedury. Również u ludzi niedokrwienie mięśnia sercowego na skutek zamknięcia proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej skutkuje większym obszarem martwicy i większą śmiertelnością w porównaniu z innymi miejscami okluzji.

Przeprowadzone w ramach pracy doktorskiej badania miały na celu określenie, na podstawie analizy markerów stresu oksydacyjnego, kardioprotekcyjnego działania kwasu acetylosalicylowego podanego dowieńcowo podczas ostrego niedokrwienia mięśnia sercowego, uzyskanego poprzez okluzję proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej u świń. Ponadto, poprzez modyfikację procedury znieczulenia

zastosowanej podczas wywołania niedokrwienia, dążono do uzyskanie stabilnego modelu niedokrwienia i zawału mięśnia sercowego o wysokiej przeżywalności zwierząt. Zamysłem badań była również charakterystyka komorowych zaburzeń rytmu serca podczas niedokrwienia mięśnia sercowego wywołanego przez okluzję proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej u świń.

Zrealizowane badania wykazały istotne statystycznie różnice dotyczące stężeń i aktywności markerów stresu oksydacyjnego u świń, którym podawano dowieńcowo kwas acetylosalicylowy, w porównaniu z grupą kontrolną zwierząt. Wykazano, że wartości całkowitej zdolności antyoksydacyjnej w tkankach pobranych z mięśnia sercowego i surowicy świń poddanych procedurze niedokrwienia mięśnia sercowego są wyższa w grupie świń otrzymujących kwas acetylosalicylowy w porównaniu z grupą kontrolną. Dowiedziono również, że wartości całkowitego statusu oksydacyjnego, wskaźniku stresu oksydacyjnego i stężenia lipofuscyny w surowicy są wyższe u zwierząt z grupy kontrolnej w porównaniu ze świniami, którym podawano kwas acetylosalicylowy. Badania wykazały ponadto, że wartości całkowitego statusu oksydacyjnego, wskaźniku stresu oksydacyjnego, stężenia dialdehydu malonowego oraz aktywności peroksydazy glutationowej, reduktazy glutationowej i S-transferazy glutationowej w tkankach pobranych z mięśnia sercowego świń są wyższe w grupie kontrolnej w porównaniu z grupą świń otrzymujących kwas acetylosalicylowy. Wykazano również, że okluzja proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej skutkuje wysoką wywoływalnością komorowych zaburzeń rytmu serca, zwłaszcza częstoskurczu komorowego i migotania komór. Udowodniono także, że modyfikacja schematu znieczulenia świń prowadzi do 100 % przeżywalności zwierząt poddanych procedurze wywołania niedokrwienia i zawału mięśnia sercowego.

Uzyskane wyniki udowodniły kardioprotekcyjne działanie kwasu acetylosalicylowego podawanego dowieńcowo w trakcie niedokrwienia i zawału mięśnia sercowego, związane ze zmniejszeniem nasilenia stresu oksydacyjnego w surowicy i tkankach mięśnia sercowego badanych świń. Przeprowadzone badania umożliwiły opracowanie stabilnego świńskiego modelu niedokrwienia i uszkodzenia niedokrwienno-reperfuzyjnego mięśnia sercowego, który cechuje się wysoką wywoływalnością arytmii komorowych i 100% przeżywalnością. Uzyskane wyniki mogą stanowić podstawę dalszych badań dotyczących dowieńcowego podawania kwasu acetylosalicylowego, zwłaszcza podczas epizodów ostrego niedokrwienia mięśnia sercowego.

Słowa kluczowe: niedokrwienie mięśnia sercowego, zawał mięśnia sercowego, uszkodzenie niedokrwienno-reperfuzyjne, kwas acetylosalicylowy, arytmie komorowe, stres oksydacyjny, świnia

ABSTRACT

Myocardial ischemia and myocardial infarction as well as other cardiovascular diseases are listed among the leading causes of human death in industrialized countries. Myocardial infarction, along with ischemic heart disease, is the most common cause of heart failure, a condition in which the heart is unable to supply an adequate amount of blood to the body's organs. Among the most significant complications of ischemia and myocardial infarction is the development of cardiac arrhythmias, especially the life-threatening ventricular arrhythmias: ventricular tachycardia and ventricular fibrillation.

The pathophysiology of myocardial ischemia is complex, and human medicine points to atherosclerosis and the atherosclerotic plaques that form in its course, which, when detached, can lead to occlusion of the lumen of the coronary vessels, resulting in myocardial ischemia, as its main cause. The most common site of occlusion of the vessels supplying the heart is the anterior descending branch of the left coronary artery. However, restoring circulation to the ischemic area of the myocardium can result in further myocardial damage known as ischemia-reperfusion injury. Its pathogenesis is complex, and among the potential processes involved in the development of ischemia-reperfusion injury are the activation and aggregation of platelets along with the sequelae of these phenomena, as well as increased production of reactive oxygen species, which additionally leads to an oxidative damage.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the action of the body's antioxidant mechanisms. Reactive oxygen species can be formed from both endogenous and exogenous sources, and their excessive production and accumulation leads to damage to cells, tissues, proteins, lipids and DNA. The most common reactive oxygen species include superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet oxygen. Non-enzymatic and enzymatic antioxidant mechanisms are used to protect the body from reactive oxygen species. Oxidative stress is one of the causes of cardiovascular disease, and its role has been confirmed in the pathogenesis and progression of

atherosclerosis, ischemia and heart failure, among others. Enzymatic and non-enzymatic markers are used to assess the severity of oxidative stress.

One of the antiplatelet drugs used in the prevention and treatment of myocardial ischemia and infarction is acetylsalicylic acid, commonly known as aspirin. In addition to inhibiting platelet activation, it exhibits anti-inflammatory, analgesic and antipyretic effects. As the main mechanism of acetylsalicylic acid's cardioprotective effect is its anti-aggregative action against platelets but the antioxidant potential of aspirin is also suggested. Acetylsalicylic acid in humans with myocardial ischemia and infarction is administered orally or intravenously, and a new concept is its intracoronary administration during an episode of acute myocardial ischemia to reduce post-reperfusion myocardial damage. The possibility of using the intracoronary route of administration to make acetylsalicylic acid more effective in myocardial ischemia is supported by the literature, where it is indicated that intracoronary than intravenous administration of other antiplatelet agents is more effective.

Research on the pathophysiology, diagnosis or treatment of myocardial ischemia and infarction uses animal models of heart disease, especially the porcine model of myocardial ischemia and infarction. The pig, due to its human-like cardiac anatomy, coronary circulation or arrhythmogenic mechanisms, is a good model of myocardial ischemia in humans. Typically, this model is achieved by transcatheter coronary vessel occlusion, including the use of angioplasty balloons. The most commonly occluded vessel is the left anterior descending branch of the left coronary artery, but occlusion of its proximal segment is rarely performed, which is associated with high animal mortality during such a procedure. Also in humans, myocardial ischemia due to occlusion of the proximal segment of the anterior descending branch of the left coronary artery results in a larger area of necrosis and higher mortality compared to other sites of occlusion.

The research was aimed at determining the cardioprotective effect of intracoronary acetylsalicylic acid administration during acute myocardial ischemia, achieved by occluding the proximal segment of the anterior descending branch of the left coronary artery in pigs. In addition, by modifying the anaesthetic protocol used during induction of ischemia, a stable model of myocardial ischemia and infarction with high animal survival was sought. The intention of the study was also to characterize ventricular arrhythmias during myocardial ischemia induced by occlusion of the proximal segment of the anterior descending branch of the left coronary artery in pigs.

The performed studies showed statistically significant differences regarding the concentrations and activities of oxidative stress markers in pigs that received intracoronary administration of acetylsalicylic acid, compared to the control group of animals. The values of total antioxidant capacity in tissues collected from the myocardium and serum of pigs undergoing myocardial ischemia procedure were shown to be higher in the group of pigs receiving acetylsalicylic acid compared to the control group. It also proved that the values of total oxidative status, oxidative stress index and serum lipofuscin concentration are higher in the control group animals compared to the pigs receiving acetylsalicylic acid. The study also showed that the values of total oxidative status, oxidative stress index, malondialdehyde concentration, and glutathione peroxidase, glutathione reductase and glutathione S-transferase activities in samples collected from porcine heart muscle are higher in the control group compared to the group of pigs receiving acetylsalicylic acid. It has also been demonstrated that occlusion of the proximal segment of the anterior descending branch of the left coronary artery results in a high inducibility of ventricular arrhythmias, especially ventricular tachycardia and ventricular fibrillation. It has also been proven that modification of the porcine anesthesia scheme leads to a 100% survival rate of animals undergoing the procedure to induce ischemia and myocardial infarction.

The results proved the cardioprotective effect of acetylsalicylic acid administered intracoronary during myocardial ischemia and infarction, associated with a reduction in the severity of oxidative stress in the serum and myocardial tissues of the studied pigs. The conducted studies enabled the development of a stable porcine model of myocardial ischemia and ischemia-reperfusion injury, which has a high inducibility of ventricular arrhythmias and 100% survival rate. The results may provide a basis for further studies on intracoronary administration of acetylsalicylic acid, especially during episodes of acute myocardial ischemia.

Keywords: myocardial ischemia, myocardial infarction, ischemia-reperfusion injury, acetylsalicylic acid, ventricular arrhythmias, oxidative stress, swine

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2. Frydrychowski, P., Michałek, M., Bil-Lula, I., Chełmecka, E., Kafel, A., Noszczyk-Nowak, A., & Stygar, D. (2022). Cardioprotective Effect of Acetylsalicylic Acid in the Myocardial
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WSTĘP

1. Ostre niedokrwienie mięśnia sercowego i zawał mięśnia sercowego

Choroby sercowo-naczyniowe, w tym ostre niedokrwienie mięśnia sercowego i zawał mięśnia sercowego (ang. *myocardial infarction*, MI), stanowią jedną z głównych przyczyn zgonów ludzi w społeczeństwach krajów uprzemysłowionych (1). Szacuje się, że MI jest odpowiedzialny za rozwój około 1/3 przypadków niewydolności serca (2) i w połączeniu z chorobą niedokrwienną serca stanowi jej najczęstszą przyczynę. Według statystyk, MI odpowiada za 10 % zejść śmiertelnych wśród osób cierpiących na choroby sercowo-naczyniowe (3).

Klinicznie, MI definiowany jest jako ostre uszkodzenie mięśnia sercowego, stwierdzone na podstawie nieprawidłowych stężeń biomarkerów sercowych, któremu towarzyszą objawy ostrego niedokrwienia mięśnia sercowego, takie jak dyskomfort i ból w klatce piersiowej, duszność i typowe zmiany stwierdzone w badaniu EKG (4).

Patofizjologia niedokrwienia i zawału mięśnia sercowego jest złożona. W medycynie człowieka za główną przyczynę MI uznaje się miażdżycę, w której przebiegu może dojść do pęknięcia i oderwania powstałych blaszek miażdżycowych, które stają się wtedy materiałem zatorowym zamykającym światło jednej z tętnic wieńcowych. Skutkiem okluzji naczynia wieńcowego jest niedokrwienie obszaru mięśnia sercowego zaopatrywanego przez daną tętnicę wieńcową. W wyniku powstałego niedokrwienia, ze względu na nieefektywną fosforylację oksydacyjną i zmniejszoną produkcję ATP przez kardiomiocyty, dochodzi do zaburzeń w metabolizmie tlenowym komórek. Konsekwencjami tych procesów jest zużycie ATP i przejście komórek na metabolizm beztlenowy, co wiąże się z nagromadzeniem w kardiomiocytach jego produktów (5). W następstwie tych procesów dochodzi do zwiększenia produkcji reaktywnych form tlenu oraz azotu, które stymulują stres oksydacyjny (6). Na skutek niedokrwienia mięśnia sercowego dochodzi do zmniejszenia objętości wyrzutowej oraz spadku ciśnienia tętniczego krwi, co prowadzi do aktywacji neurohormonalnych mechanizmów kompensacyjnych. Innymi konsekwencjami zaburzeń pracy serca jest pobudzenie baroreceptorów oraz aktywacja układu współczulnego, nasilana przez towarzyszący ból. Zmiany te skutkują zwiększeniem zapotrzebowania mięśnia sercowego na tlen, pogłębieniem niedotlenienia mięśnia sercowego oraz powiększeniem obszaru martwicy,

w efekcie czego dochodzi do rozwoju zaburzeń rytmu serca i dalszego upośledzenia funkcji serca (3).

Najczęstszym miejscem okluzji jest gałąź przednia zstępująca lewej tętnicy wieńcowej (LAD). Z dostępnej literatury dotyczącej MI u ludzi wynika, że okluzja LAD w jej odcinku proksymalnym skutkuje większym obszarem martwicy i większą śmiertelnością w porównaniu do zamknięcia światła LAD w odcinku środkowym czy dalszym (7, 8).

1.1. Uszkodzenie niedokrwienno-reperfuzyjne

Bardzo istotne w skutecznym sposobie ograniczenia strefy martwicy następującej po niedokrwieniu mięśnia sercowego jest szybkie przywrócenie perfuzji w niedokrwionym obszarze, jednak w okresie reperfuzji i reoksygenacji mięśnia sercowego dochodzi do jego dalszych uszkodzeń. Zjawisko to jest określane jako uszkodzenie niedokrwienno-reperfuzyjne mięśnia sercowego.

Mechanizm powstawania uszkodzenia niedokrwienno-reperfuzyjnego mięśnia sercowego jest wielopłaszczyznowy i dotychczas nie został w pełni poznany. Uważa się, że głównymi procesami odpowiedzialnymi za uszkodzenie niedokrwienno-reperfuzyjne są aktywacja procesów immunologicznych i zapalnych (zwłaszcza związanych z aktywacją neutrofili), zaburzenia funkcji śródbłonka, zmiany potencjału błonowego mitochondriów, zwiększona generacja reaktywnych form tlenu (ang. *reactive oxygen species,* ROS), zaburzenia wewnątrzkomórkowej gospodarki wapnia, wzmożone wytwarzanie tlenku azotu oraz zaburzenia w mikrokrążeniu i aktywacja płytek krwi prowadząca do ich agregacji i tworzenia mikrozakrzepów (9, 10). W okresie reperfuzji, na skutek napływu krwi bogatej w tlen do miejsca objętego niedokrwieniem, dochodzi do aktywacji i nasilenia wymienionych mechanizmów oraz dalszego uszkodzenia mięśnia sercowego i martwicy kardiomiocytów, również tych nieuszkodzonych w trakcie niedokrwienia, co prowadzi do powiększenia strefy zawału.

1.2. Komorowe zaburzenia rytmu serca w przebiegu MI

Istotnym powikłaniem niedokrwienia i okresu reperfuzji są zaburzenia rytmu serca, zwłaszcza arytmie komorowe, w tym zagrażające życiu tachykardia komorowa (ang.

ventricular tachycardia, VT) i migotanie komór (ang. *ventricular fibrillation*, VF). Arytmie te występują zazwyczaj we wczesnym etapie niedokrwienia, jednak proces reperfuzji również znacząco wpływa na możliwość wystąpienia komorowych zaburzeń rytmu. Zgodnie z doniesieniami literaturowymi szacuje się, że w okresie reperfuzji co najmniej jeden rodzaj arytmii rozwija się u 80% pacjentów z ostrym niedokrwieniem mięśnia sercowego (11).

Patogeneza zaburzeń rytmu serca w przebiegu MI jest wieloczynnikowa. Na skutek niedotlenienia spowodowanego niedokrwieniem dochodzi do zmniejszenia produkcji ATP podczas fosforylacji oksydacyjnej i w konsekwencji aktywacji mechanizmów glikolizy beztlenowej, której produkty gromadząc się w komórkach prowadzą do rozwoju kwasicy metabolicznej. W kwaśnym środowisku na skutek aktywacji kanałów jonowych dochodzi do pęcznienia komórek i zwiększonego gromadzenia w nich wapnia z towarzyszącym gromadzeniem się pozakomórkowo potasu, katecholamin oraz lizofosfatydylocholiny. Konsekwencją tych zmian jest depolaryzacja błony komórkowej miocytów i zmniejszenie szybkiego dokomórkowego prądu sodowego z równoczesnym zwiększeniem późnego prądu sodowego prowadzące do wydłużenia czasu trwania potencjału czynnościowego. Skutkiem dalszych zmian dotyczących dokomórkowych prądów wapniowych i odkomórkowych prądów potasowych jest skrócenie czasu trwania potencjału czynnościowego podczas niedokrwienia, zmniejszenie przezbłonowego potencjału spoczynkowego oraz zaburzenie gospodarki wewnątrzkomórkowego wapnia. Przeładowanie komórek jonami wapnia prowadzi do spontanicznych oscylacji jonów wapnia, co wyzwala indukowane po depolaryzacji wczesne i późne ektopowe pobudzenia komorowe (12, 13). Uważa się ponadto, że zawiązywanie przetrwałego monomorficznego częstoskurczu komorowego następuje w mechanizmie re-entry w obrębie tkanki bliznowatej powstałej na skutek zawału (14). Wśród innych możliwych mechanizmów odpowiadających za rozwój arytmii komorowych wymienia się również ogniska arytmogenne zlokalizowane we włóknach Purkinyego nieuszkodzonych podczas zawału, które dodatkowo charakteryzują się skróconym czasem trwania potencjału czynnościowego lub zmniejszoną amplitudą czy też zdepolaryzowanym potencjałem błonowym i zmniejszoną prędkością przewodzenia. Za miejsca szczególnie podatne na arytmogenezę uważa się okolice zawału lub tzw. strefę graniczną (12, 13).

Jednymi z najgroźniejszych powikłań MI są częstoskurcz komorowy i migotanie komór. Częstoskurczem (tachykardią) komorowym nazywamy rytm serca pochodzenia pozazatokowego, wywodzący się z mięśniówki komór, którego częstość przekracza

fizjologiczną częstość rytmu charakterystyczną dla człowieka lub danego gatunku zwierzęcia. Tachykardia komorowa jest określana jako monomorficzna, gdy zespoły komorowe mają taki sam kształt, oraz polimorficzna (wielokształtna), gdy ich morfologia jest zmienna. VT trwający dłużej niż 30 s nazywamy utrwalonym. Migotanie komór charakteryzuje się szybkimi i niezorganizowanymi skurczami komór, co w zapisie EKG jest widoczne w postaci niemiarowych, różnokształtnych zespołów komorowych. Leczenie tych zaburzeń obejmuje postępowanie farmakologiczne lub defibrylację.

1.3. Świnia jako zwierzęcy model ostrego niedokrwienia i zawału mięśnia sercowego

Wykorzystanie modeli zwierzęcych nadal odgrywa znaczącą rolę w badaniach naukowych dotyczących MI umożliwiając prowadzenie doświadczeń z zakresu patofizjologii, prewencji, diagnostyki i terapii MI.

Ze względu na zbliżoną anatomię serca i strukturę krążenia wieńcowego świni do serca ludzkiego, świński model stanowi dobry obiekt badawczy dotyczący patofizjologii i leczenia niedokrwienia i zawału mięśnia sercowego u ludzi (15, 16, 17, 18, 19, 20). Dodatkowym atutem wykorzystania tych zwierząt jest podobna masa ciała i parametry życiowe takie, jak częstość rytmu serca (21, 22, 23). Ze względu na podobieństwa dotyczące czasu trwania i kształtu potencjału czynnościowego oraz profili kanałów jonowych duże zwierzęta stanowią dobry materiał do badań dotyczących arytmii (24).

W następstwie niedokrwienia mięśnia sercowego u świń wywołanego poprzez okluzję tętnic wieńcowych uzyskuje się obraz zmian podobny do zmian patologicznych opisywanych u ludzi z MI, co dodatkowo przemawia za wykorzystaniem modelu świńskiego. Na łatwość w uzyskaniu niedokrwienia i zawału oraz będących jego następstwem zaburzeń rytmu serca wpływ ma również niewielki stopień rozgałęzienia unaczynienia wieńcowego serca świni, który znacznie utrudnia rozwój krążenia obocznego oraz wrażliwy na niedokrwienie i niedotlenienie układ bodźcoprzewodzący (25, 26, 27, 28, 29). Cechy te powodują, że okluzja LAD prowadzi do powstania stosunkowo rozległych stref zawału i różnych rodzajów arytmii (30, 31).

Świński model niedokrwienia i zawału mięśnia sercowego uzyskiwano najczęściej przez okluzję naczyń wieńcowych przeprowadzoną przeznaczyniowo lub poprzez otwarcie

klatki piersiowej. W modelach z otwartą klatką piersiową zamknięcie światła naczyń wieńcowych przeprowadzano poprzez chirurgiczne podwiązanie LAD lub gałęzi okalającej lewej tętnicy wieńcowej (LCX) (32, 33, 34, 35), zamknięcie światła LAD z wykorzystaniem zaciskaczy ameroidowych (36) lub zacisków śrubowych (37) oraz podwiązanie gałęzi skośnych LAD oraz uszkodzenie ściany wolnej lewej komory (38). Modele niedokrwienia i zawału prowadzone z otwarciem klatki piersiowej, poprzez bezpośredni dostęp do serca, umożliwiają bezpośrednią kontrolę nad prowadzonym zabiegiem okluzji tętnic wieńcowych. Jednak ich uzyskanie wymaga zabiegów takich, jak torakotomia czy nacięcie worka osierdziowego, które zwiększają ryzyko rozwoju infekcji (39) i mogą wpływać na rozmiar zawału, reakcję zapalną oraz przebudowę mięśnia sercowego po niedokrwieniu (40, 41, 42). Dodatkowo, zabiegi prowadzone w celu uzyskania modeli z otwartą klatką piersiową mogą mieć wpływ na homeostazę organizmu i jego regenerację (39). Wszystkie te elementy wpływają na wyższą śmiertelność i częstość powikłań (43) tych modeli. Wartym odnotowania jest również fakt, iż MI wywołane w modelach z otwarciem klatki piersiowej różnią się patofizjologicznie od MI u ludzi, do którego dochodzi najczęściej na skutek oderwania blaszki miażdżycowej prowadzącego do zamknięcia światła tętnicy wieńcowej (44).

Uzyskanie modelu MI bez konieczności otwarcia klatki piersiowej pozwala na wyeliminowanie wielu z wymienionych problemów. W technikach przeprowadzanych u świń z zamkniętą klatką piersiową MI uzyskiwano przez okluzję tętnic wieńcowych z wykorzystaniem balonu angioplastycznego (45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62), coili (63, 64), kulek z żelu agarozowego (25) lub specjalnie wykonanych gąbek piankowych (65) czy też kulek z dołączonym włóknem (66). Zdecydowaną zaletą modeli z zamkniętą klatką piersiową jest ich mniejsza inwazyjność w porównaniu do modeli z otwartą klatką piersiową, co wpływa na zwiększenie przeżywalności zwierząt a także - w większości przypadków – skrócenie czasu procedur i ograniczenie ich kosztów (43).

W modelach wykorzystujących balony angioplastyczne ostre niedokrwienie mięśnia sercowego uzyskiwano poprzez okluzję balonem LAD (26, 46, 47, 48, 49, 50, 51, 52, 53, 59, 60, 61, 62, 67) lub LCX (50, 51, 54). Najczęściej zamknięcie LAD przeprowadzano w jej środkowej części (30, 47, 53, 55, 67), głównie dystalnie do pierwszej gałęzi przegrodowej (46) czy też gałęzi diagonalnej (50, 51, 52, 56, 57, 58). Miejscem zamknięcia LCX zazwyczaj był jej odcinek początkowy (50, 51) lub dalszy (54). Taki wybór miejsc okluzji tętnic wieńcowych pozwala na zwiększenie przeżywalności zwierząt wykorzystywanych w doświadczeniu. W większości świńskich modeli MI nie wywołuje się okluzji LAD w jej

proksymalnym odcinku. Zamknięcie światła LAD w jej początkowej części skutkuje większym obszarem wywołanego niedokrwienia i może wpływać na zmniejszenie przeżywalności zwierząt m.in. poprzez rozwój zaburzeń rytmu serca, głównie w postaci migotania komór, i zaburzeń hemodynamicznych (30, 35, 58).

2. Kwas acetylosalicylowy i jego zastosowanie w ostrym niedokrwieniu i zawale mięśnia sercowego

W ostatnich latach postuluje się udział płytek krwi jako jednego z głównych czynników mających wpływ na rozwój uszkodzenia niedokrwienno-reperfuzyjnego (9, 68, 69). Na skutek aktywacji płytek krwi dochodzi do ich agregacji i powstawania mikrozakrzepów, które lokalizując się w drobnych naczyniach krwionośnych w obrębie mięśnia sercowego prowadzą do jego uszkodzenia (9). Ponadto po aktywacji płytki krwi uwalniają swoją zawartość w postaci mikrocząsteczek (ang. *microvesicles*), egzosomów i ciałek apoptycznych, które nasilają stan zapalny w obrębie niedokrwionego mięśnia sercowego (9). Proces zapalny jest dodatkowo wzmacniany przez agregację płytek krwi i leukocytów prowadzącą do napływu leukocytów do miejsca niedokrwienia (70). Produkowane przez trombocyty wazokonstryktory, m.in. tromboksan A2, nasilają dysfunkcję śródbłonka poprzez silne działanie zwężające naczynia krwionośne (71). Jako jeden z mechanizmów uszkadzających mięsień sercowy wymienia się również pobudzenie włókien czuciowych nerwów rdzeniowych, które może skutkować charakterystycznym bólem w klatce piersiowej, nadciśnieniem tętniczym, rozwojem tachyarytmii (72) i zwiększeniem zapotrzebowania mięśnia sercowego na tlen (9). Jedną z substancji o działaniu przeciwpłytkowym najczęściej wykorzystywaną w leczeniu i prewencji MI jest kwas acetylosalicylowy (ang. *acetylsalicylic acid*, ASA), popularnie zwany aspiryną. Jest to lek o działaniu przeciwzapalnym, przeciwbólowym i przeciwgorączkowym oraz hamującym aktywność płytek krwi. Działanie ASA polega na inhibicji cyklooksygenazy, która jest katalizatorem w reakcji syntezy prostaglandyn, prostacyklin i tromboksanów, określanych jako mediatory stanu zapalnego. Szczególnie istotne jest działanie tromboksanu A2, który nasila aktywację i agregację płytek krwi. ASA nieodwracalnie hamuje obie izoformy cyklooksygenazy, głównie jednak COX 1, co poprzez zahamowanie syntezy tromboksanu A2 prowadzi do zablokowania możliwości aktywacji i agregacji trombocytów (73). To właśnie działanie antyagregacyjne kwasu acetylosalicylowego jego wskazywane jako główny mechanizm jego działania kardioprotekcyjnego. ASA wykazuje u chorych z MI również

działanie przeciwzapalne i minimalizuje dysfunkcję śródbłonka poprzez zablokowania czynników o działaniu obkurczającym naczynia krwionośne (71, 74). Sugeruje się również antyoksydacyjny potencjał ASA jako jeden z mechanizmów działania kardioprotekcyjnego tego leku.

Dotychczas u chorych z ostrym niedokrwieniem i zawałem mięśnia sercowego kwas acetylosalicylowy jest podawany doustnie lub dożylnie, jednak ze względu na dużą popularność i dostępność tego leku trwają poszukiwania jego skuteczniejszego zastosowania. Jednym z nowych pomysłów jest dowieńcowe podanie ASA podczas ostrego niedokrwienia, co ma na celu zmniejszenie uszkodzenia poreperfuzyjnego mięśnia sercowego. Potrzeba przetestowania tej koncepcji na zwierzętach oparta jest na badaniach obserwacyjnych u ludzi, u których znane jest zjawisko oporności na aspirynę, polegające na braku działania ochronnego ASA przed powikłaniami zatorowo-zakrzepowymi (75, 76, 77). Ponadto wykazano, że uszkodzenie poreperfuzyjne oceniane pogorszeniem przepływu w mikrokrążeniu wieńcowym częściej obserwowano u pacjentów z niepełną odpowiedzią na ASA podaną doustnie lub dożylnie (78). Przezskórne interwencje wieńcowe (ang. *percutaneous coronary interventions*, PCI), które są preferowaną metodą uzyskania reperfuzji u pacjentów z MI, umożliwiają precyzyjne podanie ASA do niedokrwionego obszaru bez konieczności wykonywania innych dodatkowych procedur naczyniowych, np. w celu administracji leku.

Jak dotąd nie opisano dowieńcowej podaży kwasu acetylosalicylowego, jednak doniesienia literaturowe, wskazujące na większą skuteczność dowieńcowego niż dożylnego podania innych substancji o działaniu przeciwpłytkowym, głównie inhibitorów glikoproteiny IIb/IIIa (79, 80, 81), pozwalają przypuszczać, że dowieńcowe podanie ASA może być skuteczniejsze w poprawie przepływu wieńcowego w obszarze niedokrwienia w porównaniu z podaniem doustnym lub dożylnym.

3. Stres oksydacyjny

Stres oksydacyjny jest opisywany jako brak równowagi pomiędzy produkcją i nagromadzeniem reaktywnych form tlenu (ROS) w komórkach i tkankach ustroju a zdolnością organizmu do unieczynniania tych reaktywnych produktów (82).

Fizjologicznie ROS biorą udział w niektórych procesach toczących się w organizmach żywych, takich, jak sygnalizacja komórkowa i powstają jako produkty uboczne przemian tlenu w organizmie. Jednakże ich nadmierna produkcja, zaburzając równowagę, prowadzi do uszkodzeń komórek i tkanek oraz białek, lipidów i DNA. Wolne rodniki powstają zarówno ze źródeł o charakterze endogennym, jak i egzogennym. Źródłami wewnętrznymi mogą być procesy zapalne i nowotworowe, niedokrwienie a także choroby, takie, jak miażdżyca tętnic wieńcowych czy procesy starzenia się organizmu. Z kolei do źródeł zewnętrznych wolnych rodników zalicza się m.in. metale ciężkie, toksyny czy leki.

W celu ochrony przed reaktywnymi formami tlenu w organizmie uruchamiane są mechanizmy obronne, które są oparte głównie o reakcje enzymatyczne z udziałem takich enzymów, jak peroksydaza glutationowa, reduktaza glutationowa, S-transferaza glutationowa czy katalaza i dysmutaza ponadtlenkowa. Ponadto znane są związki biologiczne wykazujące działanie ochronne przed ROS, stanowiące tzw. nieenzymatyczne mechanizmy antyoksydacyjne. Wśród nich można wymienić karotenoidy, flawonoidy, polifenole, witaminy A, C i E, kwas moczowy czy glutation.

Najczęstszymi reaktywnymi formami tlenu są anionorodnik ponadtlenkowy ($O_2^{\bullet -}$), nadtlenek wodoru (H_2O_2), rodnik hydroksylowy ($HO^{\bullet}$) oraz tlen singletowy ($^1O_2$). Głównym miejscem produkcji ROS są mitochondria, a ich generowanie odbywa się zarówno w warunkach fizjologicznych, jak i patologicznych. Reaktywne formy tlenu mogą być wytwarzane podczas oddychania komórkowego, metabolizmu kwasu arachidonowego czy też przez komórki śródbłonka i komórki zaangażowane w procesy zapalne (83).

3.1. Wpływ stresu oksydacyjnego na układ sercowo-naczyniowy

Badania dowodzą, że stres oksydacyjny i reaktywne formy tlenu mogą być zaangażowane w powstawanie i progresję wielu chorób, wśród których można wymienić

choroby nowotworowe, cukrzycę i inne zaburzenia metaboliczne a także miażdżycę oraz choroby układu sercowo-naczyniowego (84).

Udowodniono, że stres oksydacyjny należy rozpatrywać jako jedną z pierwotnych lub wtórnych przyczyn wielu chorób układu sercowo-naczyniowego (85). Liczne badania potwierdzają rolę stresu oksydacyjnego w patogenezie i progresji miażdżycy, niedokrwienia, nadciśnienia tętniczego, kardiomiopatii, przerostu mięśnia sercowego oraz zastoinowej niewydolności serca (86, 87, 88).

Stres oksydacyjny przyczynia się do rozwoju przewlekłej niewydolności serca poprzez aktywację szlaków odpowiedzialnych za patologiczną przebudowę mięśnia sercowego, upośledzenie funkcji skurczowej, stymulację fibroblastów i aktywację metaloproteinaz (89).

Istotne, zwłaszcza w kontekście ostrego niedokrwienia mięśnia sercowego, jest działanie stresu oksydacyjnego jako czynnika inicjującego rozwój miażdżycy. Wczesne zapalenie śródbłonka, będące przyczyną tworzenia blaszki miażdżycowej, skutkuje produkcją ROS przez makrofagi rekrutowane *in situ*. Powstałe w ten sposób reaktywne formy tlenu utleniają krążące we krwi lipoproteiny o małej gęstości (ang. *low-density lipoprotein,* LDL), a wynikiem tych procesów jest powstanie blaszki miażdżycowej (82).

Ostre niedokrwienie mięśnia sercowego, zwłaszcza po okresie reperfuzji, prowadzi do wytwarzania w mięśniu sercowym reaktywnych form tlenu, które uszkadzając błonę komórkową miocytów prowadzą do ich śmierci (90). Ponadto podczas niedokrwienia enzymy antyoksydacyjne, znajdujące się w mitochondriach i cytozolu miocytów, tracą swoją funkcję i przedostają się do płynu pozakomórkowego, skąd są „wypłukiwane" w trakcie reperfuzji. Proces ten upośledza zdolność organizmu do kontroli generowania wolnych rodników, a ich niekontrolowana produkcja przekracza możliwości enzymów antyoksydacyjnych, prowadząc do dalszego, nieopanowanego wytwarzania ROS (91). Mechanizmy te mają swoje potwierdzenie w badaniach naukowych. Na modelu szczurzym wykazano, że zawał mięśnia sercowego i rozwijająca się niewydolność serca są powiązane z nasileniem stresu oksydacyjnego i osłabieniem zdolności antyoksydacyjnej (92).

Dane literaturowe potwierdzają korzystne działanie antyoksydantów na rozwój, progresję i przebieg chorób serca, zwłaszcza tych, których patogeneza jest związana z

nadmiernym stresem oksydacyjnym (93). Udowodnionym skutkiem działania substancji o działaniu przeciwutleniającym jest ograniczenie patologicznej przebudowy przedsionków indukowanej szybkim rytmem serca oraz poprawa funkcji śródbłonka u pacjentów z miażdżycą (94, 95). Znany jest również terapeutyczny wpływ antyoksydantów na progresję niewydolności serca (96).

3.2. Markery stresu oksydacyjnego

Markerami stresu oksydacyjnego nazywamy wskaźniki, których oznaczenie i analiza pozwala na ocenę nasilenia stresu oksydacyjnego. Dobry marker powinien cechować się odpowiednią specyficznością i czułością w odniesieniu do zmian stężeń ROS, stabilnością w czasie oraz powtarzalnością pomiarów. Wyróżnić możemy enzymatyczne i nieenzymatyczne markery stresu oksydacyjnego. W części badawczej niniejszej pracy jako markery stresu oksydacyjnego wykorzystano całkowitą zdolność antyoksydacyjną (ang. *total antioxidant capacity*, TAC), całkowity status oksydacyjny (ang. *total oxidative status*, TOS), wskaźnik stresu oksydacyjnego (ang. *oxidative stress index*, OSI), stężenie dialdehydu malonowego (ang. *malondialdehyde*, MDA), aktywność peroksydazy glutationowej (ang. *glutathione peroxidase*, GPx), aktywność reduktazy glutationowej (ang. *glutathione reductase*, GR), aktywność S-transferazy glutationowej (ang. *glutathione S-transferase*, GST) oraz stężenie lipofuscyny (ang. *lipofuscin*, LF).

3.2.1. Nieenzymatyczne markery stresu oksydacyjnego

3.2.1.1. Całkowita zdolność antyoksydacyjna, całkowity status oksydacyjny i wskaźnik stresu oksydacyjnego

Całkowita zdolność antyoksydacyjna (TAC) służy do całościowej oceny aktywności przeciwrodnikowej badanych próbek i jest wykorzystywana do określenia zdolności antyoksydacyjnych danego materiału, czyli jego potencjału w minimalizowaniu stresu oksydacyjnego. W pomiarach TAC w warunkach *in vitro* stosuje się metody wykorzystujące reakcje między antyoksydantami a modelowymi wolnymi rodnikami (np. ABTS, sól diamonowa 2,2’-azobis(3-etylobenzotiazolino-6-sulfonianu)) czy jonami metalu lub techniki polegające na hamowaniu peroksydacji lipidów (np. MDA).

Ocenę ogólnego statusu oksydacyjnego organizmu przeprowadza się poprzez określenie całkowitego statusu oksydacyjnego (TOS), z kolei wskaźnik stresu oksydacyjnego (OSI) to parametr pomiaru nasilenia stresu oksydacyjnego pozwalający na porównanie stosunku prooksydantów i antyoksydantów w organizmie.

3.2.1.2. Dialdehyd malonowy

Dialdehyd malonowy (MDA) jest istotnym markerem stresu oksydacyjnego, zwłaszcza peroksydacji lipidów (97). MDA stanowi jeden z końcowych produktów peroksydacji lipidów błony komórkowej, podatnych na zwiększenie stężenia ROS w stresie oksydacyjnym. Jego stężenie w organizmie w sposób bezpośredni świadczy o stopniu peroksydacji lipidów, a pośrednio odzwierciedla stopień uszkodzenia komórek. Dialdehyd malonowy jest związkiem o wysokiej reaktywności, który wykazuje działanie mutagenne i cytotoksyczne czy uszkadzające białka i cząsteczki kwasów nukleinowych.

3.2.2. Enzymatyczne markery stresu oksydacyjnego

3.2.2.1. Peroksydaza glutationowa

Peroksydaza glutationowa (GPx) jest metaloenzymem zależnym od selenu, który katalizuje rozkład nadtlenku wodoru do wody i lipidów, czemu towarzyszy przekształcenie zredukowanej formy glutationu (GSH) w jego formę utlenioną (GSSG). Peroksydaza glutationowa uważana jest za jeden z elementów pierwszej obrony antyoksydacyjnej organizmu i wpływa na ograniczenie peroksydacji lipidów. W organizmie ssaków GPx występuje w formie 8 izomerów, a największe znaczenie przypisuje się izoformom GPx-1, GPx-2, GPx-3 i GPx-4. Dzięki bliskiemu powiązaniu syntezy GPx ze stężeniem ROS w ustroju, ocena aktywności peroksydazy glutationowej jest dobrym markerem stresu oksydacyjnego (98).

3.2.2.2. Reduktaza glutationowa

Reduktaza glutationowa (GR) jest homodimerem, który katalizuje reakcje redukcji utlenionego glutationu (GSSG) do jego zredukowanej formy (GSH). Reakcja ta jest zależna od NADPH, a jej składowe – GSSG i GSH – są związkami kluczowymi w utrzymaniu

równowagi redoks ustroju. GSH bierze udział w inaktywacji reaktywnych form tlenu przy udziale S-transferazy glutationowej, która jest kolejnym enzymem ważnym w utrzymaniu homeostazy redoks organizmu. Reduktaza glutationowa współdziała z peroksydazą glutationową, której dostarcza zredukowanej formy glutationu do jego reakcji.

3.2.2.3. S-transferaza glutationowa

S-transferazy glutationowe (GST) to grupa enzymów odpowiedzialna za katalizowanie reakcji sprzęgania zredukowanej formy glutationu (GSH) z ksenobiotykami w celu ich detoksykacji. Znane są 3 izoformy GST, a ich główną rolą w ustroju jest metabolizowanie reaktywnych form tlenu i innych szkodliwych dla organizmu związków (np. kancerogenów, insektycydów i niektórych leków). Wykazano także, że GST odgrywają ważną rolę jako regulator szlaków kinazy białkowej aktywowanej mitogenami w uszkodzeniu niedokrwienno-reperfuzyjnym (99).

3.2.2.4. Lipofuscyna

Lipofuscyna (LF) jest materiałem złożonym z utlenionych reszt białkowych i lipidowych, która, nie ulegając degradacji, jest akumulowana w organizmie. Jej nagromadzenie w ustroju jest proporcjonalnie uzależnione od stopnia nasilenia produkcji reaktywnych form tlenu oraz od zmian mitochondrialnych, które zachodzą w organizmach żywych wraz z wiekiem, czyli starzeniem się organizmu (100). Lipofuscyna powstaje jako efekt reakcji produktów peroksydacji lipidów i związków zawierających część aminową.

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CELE PRACY DOKTORSKIEJ

Przeprowadzone badania miały na celu:

1. Ocenę kardioprotekcyjnego działania ASA podanego dowieńcowo podczas ostrego niedokrwienia mięśnia sercowego poprzez analizę wybranych markerów stresu oksydacyjnego:
a) w surowicy świń
b) w tkankach mięśnia sercowego świń pobranych ze strefy zawałowej oraz z obszaru nieobjętego zawałem

2. Zmodyfikowanie procedury znieczulenia stosowanej w dotychczasowych badaniach na świńskim modelu niedokrwienia i zawału mięśnia sercowego i uzyskanie stabilnego modelu o wysokiej przeżywalności zwierząt

3. Charakterystyka komorowych zaburzeń rytmu serca rozwijających się podczas niedokrwienia mięśnia sercowego na skutek okluzji proksymalnego odcinka LAD.

MANUSKRYPT I

Frydrychowski,P., Michałek,M., Kuliczkowski,W., Nowak,K., Skrzypczak,P., Bil-Lula,I. & Noszczyk-Nowak,A. (2022).The impact of a modified anaesthetic protocol on animal survival and the characteristics of ventricular arrhythmias in the course of acute myocardial infarction in a domestic pig model. Journal of Veterinary Research, 66 (3). https://doi.org/10.2478/jvetres-2022-0046

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The impact of a modified anaesthetic protocol on animal survival and the characteristics of ventricular arrhythmias in the course of acute myocardial infarction in a domestic pig model

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Abstract

Introduction: Acute myocardial infarction (MI) is one of the most common causes of death in humans in highly developed countries. Among its most frequent complications affecting the patient's prognosis are cardiac arrhythmias: ventricular tachycardia (VT) and ventricular fibrillation (VF). **Material and Methods:** The study aimed to characterise arrhythmias in 19 pigs subjected to experimentally induced MI obtained by occlusion of the proximal left anterior descending (LAD) coronary artery using an angioplasty balloon. The anaesthetic protocol was modified to reduce mortality by including procedures stabilising haemodynamic disorders which develop during episodes of ischaemia and arrhythmia. During 30 min of experimentally induced ischaemia, the heart rhythm was recorded using a 12-lead ECG. The time, frequency, and type of arrhythmias were analysed. **Results:** Ventricular arrhythmias were found in 94.74% of the treated pigs. The most common were ventricular premature complexes, reported in 88.89% of pigs with arrhythmia. Ventricular tachycardia was recorded in 66.67% and ventricular fibrillation in 50% of pigs with arrhythmias. **Conclusion:** Myocardial infarction due to proximal LAD occlusion is characterised by a high incidence of ventricular arrhythmias, especially VT and VF. Because of the high survival rate, this MI porcine model may serve as a model for research on acute ischaemic ventricular arrhythmias in humans. Additionally, it reduces the total number of animals required for testing while yielding meaningful results, which is in line with the 3R principle.

Keywords: acute myocardial infarction, domestic pig model, proximal left anterior descending coronary artery occlusion, ventricular arrhythmias.

Introduction

Abnormal biomarker levels with signs of acute myocardial ischaemia satisfy the clinical definition of myocardial infarction (MI) (54). Obstruction of one of the coronary arteries results in ischaemia of the area supplied by this artery (54). The most common site of occlusion is the left anterior descending coronary artery (LAD). Studies on MI in humans show that occlusion in the proximal section of the LAD results in a greater area of necrosis and more significant mortality than medial or distal LAD occlusion (8, 18). The common complications of MI are life-threatening arrhythmias – ventricular tachycardia (VT) and ventricular fibrillation (VF) – which are considered the leading causes of death in the acute phase of myocardial infarction (5, 17). These arrhythmias usually occur in the early stages of ischaemia, but the reperfusion process also significantly increases the possibility of their development. Possible causes include hypoxia of cells resulting in metabolic acidosis which disturb the ion balance of the cells (15, 22), arrhythmias in the re-entry

mechanism (33), or foci of cardiac arrhythmias (15, 22).

The use of animal models plays a significant role in MI research, enabling experiments in the pathophysiology, prevention, diagnosis, and therapy of MI. Generally, large animals are good material for arrhythmia studies because of similarities in the action potential and ion channel profiles (11). Since the pig heart's anatomy and size, and the structure of its coronary circulation are similar to those of the human heart, it is a suitable research model for studying the pathophysiology and treatment of human myocardial ischaemia (2, 19, 27, 31, 32, 60). Additionally, pigs have similar body weight and vital parameters to humans, such as the heart rate (24, 40, 43). Myocardial ischaemia caused by occlusion of the coronary arteries in pigs produces similar changes to the pathological changes reported in humans with MI, which further justifies using the porcine model. This feature significantly impedes the development of collateral circulation and the heart stimulatory system that is sensitive to ischaemia and hypoxia (10, 13, 17, 41, 53). In summary, LAD occlusion in the pig model leads to the formation of relatively large infarct zones and various types of arrhythmias (28, 29).

The porcine model of myocardial ischaemia is most often obtained by transvascular or thoracic occlusion of the coronary vessels. Models of MI in the pig with a closed chest include occlusion of the coronary arteries using an angioplasty balloon (9, 39, 44, 47, 51, 52, 57, 61, 62), coils (14, 21), agarose gel balls (17), a purpose-made foam sponge (46), or balls with an attached filament (36). Closed-chest models are less invasive than open-chest models and more similar to human MI pathophysiology. They are more survivable by animals and – in most cases – need shorter procedure times and cost less (37). Studies using LAD occlusion to induce MI in pigs have used different anaesthetic protocols (28, 35, 47, 51, 52). Usually, anaesthetic care consisted of appropriate premedication of the animal, then induction of general anaesthesia and its maintenance. For premedication, most often tiletamine (52) or a combination of tiletamine with zolazepam (51), ketamine (28), midazolam (35), atropine (28) or their mixtures (28, 47) were used. To induce anaesthesia, propofol (47), isoflurane (35, 51, 52), sodium pentobarbital (28) or thiopental (35) were used. The sinus rhythm was restored with appropriate drugs, such as amiodarone (28, 47, 51, 52) and lignocaine (28, 35, 47, 51) or with defibrillation (28, 35, 47, 51, 52). However, the available publications do not describe anaesthetic procedures stabilising the animal's condition and correcting the haemodynamic disorders caused by MI and the development of cardiac arrhythmias. Haemodynamic disturbances due to MI induced by LAD occlusion may be hypotension, hypovolaemia, or shock, which if left untreated may result in the death of the animal during the procedure or a shorter survival time after its completion. Appropriate action to compensate for these disorders is essential, especially in the procedure of LAD closure in its proximal part, which is characterised by high mortality (28, 35, 52).

So far, the porcine infarction model has been mainly used to study ventricular arrhythmias (39, 41, 55), antiplatelet drugs for myocardium cardioprotection (57, 61, 62), VF management (30, 39, 45, 58), post-reperfusion injuries (9) and therapies for heart failure following MI (51). However, pigs have a predisposition to ventricular arrhythmias in MI that are resistant to treatment, which results in the high mortality rate of this model in standard anaesthetic management (37, 41, 49, 59). In pig MI models generated with an angioplasty balloon, LAD occlusion leading to ischaemia is performed in the medial or distal LAD (9, 25, 28, 30, 39, 44, 45, 48, 52, 55, 57, 58), which increases the survival rate of the animals used in the experiment. To the best of our knowledge, there are no reports in the literature on an effective way to achieve MI in pigs by occluding the proximal part of the LAD using an angioplasty balloon. The high mortality associated with MI due to proximal LAD occlusion in humans recommends the development of a stable porcine MI model induced by the same LAD occlusion as good material for research on the therapeutic and protective management of the myocardium during ischaemia. Hence, the presented study aimed firstly to evaluate a porcine model of MI obtained by proximal LAD occlusion under a modified anaesthetic protocol intended to stabilise the condition of the pigs during MI and secondly to characterise the ventricular arrhythmias developing during the procedure.

Material and Methods

Animals. The study was carried out on 19 female pigs of the Polska Biała Zwisłoucha breed (National Research Institute of Animal Production Experimental Station, Żerniki Wielkie, Poland), aged 16–20 weeks and weighing 33–44 kg. The pigs were all fed an identical complete feed meeting nutritional standards and kept under the same breeding conditions. Before starting the tests, all animals underwent acclimation to become used to handling and grooming activities.

Protocol for establishing a model of acute myocardial infarction – preparation of animals. The animals' access to food was restricted for 12 h before the procedures started. All procedures and measurements were performed according to the same anaesthesia scheme and the same operating procedure.

Premedication and induction and maintenance of general anaesthesia. Pigs were premedicated with an intramuscular injection of a mixture containing ketamine (Vetaketam; Vet-Agro, Lublin, Poland) (10 mg/kg b.w.), midazolam (Midanium; Polfa Warszawa, Warsaw, Poland) (0.3 mg/kg b.w.) and medetomidine (Sedator; Eurovet Animal Health BV,

Bladel, The Netherlands) (0.03 mg/kg b.w.). The drugs used in the premedication differ from those in the schemes described in the literature (23, 30, 32, 33, 39), and to our knowledge, this combination was used for the first time. After immobilisation, the animals were placed in the sternum position, and vascular access was obtained to the marginal vein of the ear. After general anaesthesia induction, propofol (Provive; Claris Lifesciences UK, Crewe, UK) was administered intravenously (2 mg/kg b.w.), and the animal was intubated with a size 8 endotracheal tube (Murphy Tracheal Tube; SUMI, Sulejówek, Poland). After intubation, the animals were mechanically ventilated using 100% oxygen and a closed gas system with a carbon dioxide absorber. Ventilation was initiated in the pressure-swing mode, and after the animal was stabilised and the ventilation and capnometry parameters were assessed, it was switched to the volumetric-swing mode. The oxygen flow was maintained at 2 L/min (Primus; Dräger Medical AG & Co., Lübeck, Germany) with a tidal volume of 10 mL/kg b.w. and the number of breaths was 12/min. The tidal volume was appropriately adjusted to maintain the end-tidal CO_2 concentration between 35 and 45 mmHg. Anaesthesia was maintained with isoflurane (Forane; Abbott Laboratories, Warsaw, Poland) administered using an appropriately calibrated vaporiser (Vapor 2000; Dräger Medical AG & Co). The isoflurane concentration (1.5% to 2.5% in 100% oxygen) was monitored (Lifepack 12, Medtronic, Redmond, WA, USA) and regulated using a vaporiser based on capnometry values to keep the final concentration at 1 minimum alveolar concentration. Intraoperative analgesia was provided by intravenous administration of fentanyl (Fentanyl WZF; Polfa Warszawa), initially as a bolus (10 µg/kg b.w.) and then as a continuous infusion at a dose of 10 µg/kg b.w./h. During the procedure, fluid therapy was administered in the form of constant rate infusion (CRI) of a multi-electrolyte solution (Optilyte; Fresenius Kabi Polska, Kutno, Poland) at a dose of 6–12 mL/kg b.w./h, depending on the animal's hydration status and response to drugs. Throughout the procedure, complete anaesthetic monitoring was carried out by non-invasively measuring internal body temperature, saturation, pulse and blood pressure (Lifepack 12) and electrocardiography. The heart rhythm was continuously monitored with a 12-lead ECG test (BTL-08 MT Plus ECG; BTL Industries, Stevenage, UK).

Treatment of haemodynamic disorders. In the event of a drop in blood pressure during the procedure, the patients were haemodynamically stabilised. For this purpose, the animal was treated as needed with boluses of lactated Ringer's solution (Fresenius Kabi Polska) (10 mL/kg b.w.) and boluses of hydroxyethyl starch 130/0.4 (Voluven; Fresenius Kabi Deutschland, Bad Homburg vor der Höhe, Germany) (3–5 mL/kg b.w.). In the absence of a haemodynamic response to the applied fluid boluses, continuous infusion of dopamine (Dopaminum Hydrochloricum WZF; Polfa Warszawa) was started at an initial dose of 4 µg/kg b.w./min and subsequently adjusted depending on the operational needs and the animal's response to the treatment. There was no need to use dopamine at a dose above 10 µg/kg b.w./min during the procedures. The patient was put into the Trendelenburg position to increase blood flow to the heart and cardiac output until hypovolaemia was corrected.

Induction of myocardial infarction. Percutaneous access to the femoral artery was obtained under ultrasound control (F37; Hitachi Aloka Medical Ltd, Mure, Mitaka-shi, Tokyo, Japan) using a 21G femoral puncture needle (21G; Balton, Warsaw, Poland) and a 6F diameter vascular introducer sheath (Balton). Following insertion of a 6F diameter Judkins Left 3.5 curvature guide catheter (Launcher; Medtronic), heparin (Heparinum WZF; Polfa Warszawa) was administered (6,000 UI), and coronary angiography was performed (Fig. 1).

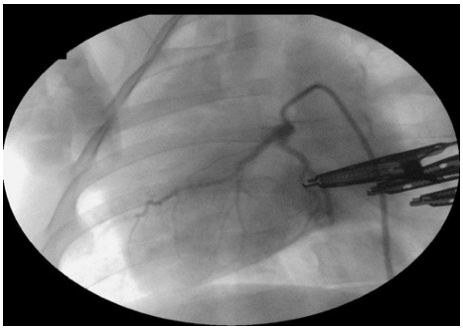

Fig. 1. Coronary angiography performed on a female pig subjected to myocardial infarction induced by 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol

Then a 0.014″ balance middleweight 300 cm angioplasty guidewire (Abbott, Santa Clara, CA, USA) was inserted through the catheter and placed under fluoroscopic control (Symbol; General Medical Merate SpA, Seriate, Italy) in the proximal segment of the LAD. A 3.0 × 10 mm over-the-wire angioplasty balloon (Sprinter; Medtronic) was placed on the guidewire. The balloon was inflated to 6 atm and held at that pressure for 30 min for complete LAD occlusion. Arterial closure was confirmed by angiography (Fig. 2), while MI was diagnosed by ST-segment elevation on the 12-lead ECG (Fig. 3). Additionally, MI was confirmed by histopathological analysis of myocardial tissues collected from the animals when euthanised 4 weeks after the procedure.

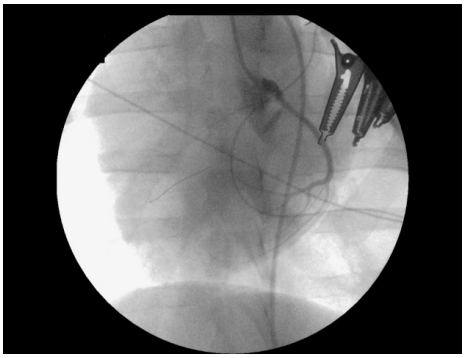

Fig. 2. Coronary angiography presented occlusion of the proximal segment of the left anterior descending artery achieved with an angioplasty balloon in a female pig subjected to myocardial infarction in a modified anaesthetic protocol

Anti-arrhythmic treatment. The VT onset was treated with an intravenous infusion of 2% lignocaine (Lignocainum Hydrochloricum WZF 2%; Polfa Warszawa) at 25–50 µg/kg b.w. (the dose was adjusted according to the animal's response to treatment) and an infusion of amiodarone (Cordarone; Sanofi-Aventis France, Paris, France) at 5 mg/kg b.w./h diluted in 250 mL of 5% glucose solution (B. Braun, Melsungen, Germany). Ventricular fibrillation was terminated with external defibrillation at 300 J or 360 J for recurrent VF episodes (Lifepack 12).

Statistical analysis. Data on the number and type of arrhythmias and their duration were statistically analysed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). The Shapiro–Wilk normality test was used to check the distribution and normality of the data distribution. The mean ± standard deviation was calculated for normally distributed data, while the median (range) was calculated for skewed data. Differences in the incidence of arrhythmias were calculated using nonparametric Kruskal–Wallis analysis followed by Dunn's multiple comparisons in a post-hoc test. A P-value of <0.05 was considered statistically significant. Charts presenting selected data were prepared in the free-to-use web wizard LiveGAP (https://charts.livegap.com).

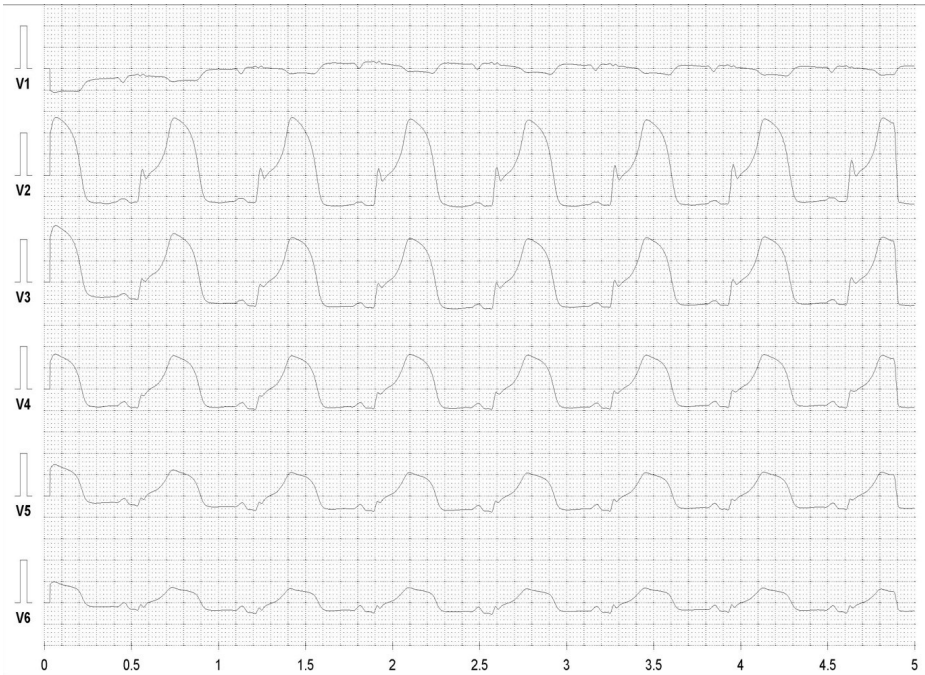

Fig. 3. Features of acute myocardial ischemia – ST-segment elevation in a female pig subjected to myocardial infarction induced by 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol. The sinus rhythm is shown at a rate of 106 bpm. V1 – costochondral junction of the right first intercostal space; V2 – sixth intercostal space, to the left of the sternum; V3 – midway point between leads V2 and V4; V4 – costochondral junction of the sixth intercostal space; V5 – sixth intercostal space, dorsal to V4 at a distance equal to the distance between V2 and V3 or V3 and V4; V6 – sixth intercostal space, dorsal to V5 at a distance equal to the distance between V2 and V3, V3 and V4 or V4 and V5. Paper speed: 50 mm/s; amplitude: 10 mm/mV; 25 Hz notch filter; Fuzzy+ software filter

Results

All 19 animals survived the 30 minutes LAD occlusion procedure, and 19 records were analysed. All pigs subjected to the procedure showed an elevated electrocardiographic ST-segment in the first 10 min of induction of LAD occlusion, with a median of 1 (1–5) min. Only one animal (5.26% of all pigs) showed no cardiac arrhythmias during an induced MI. In comparison, at least one type of arrhythmia was diagnosed in the rest of the 18 pigs (94.74% of all pigs) ($P < 0.0001$). Treatment aimed at correcting haemodynamic disturbances was administered to nine pigs which developed VF. The use of dopamine, in addition to fluid therapy, was required in seven pigs. Table 1 shows the mean values of the ECG parameters determined before, during, and after induction of MI.

Types of arrhythmias. Ventricular arrhythmias were found in all 18 animals with the described cardiac arrhythmias. The most common arrhythmia was ventricular premature complexes (VPCs) (Figs 4a and 4b), defined as a single ventricular beat occurring earlier than the expected sinus beat. It was found in the electrocardiographic records of 16 pigs (84.21% of all pigs and 88.89% of pigs with diagnosed arrhythmia). All 16 pigs with VPCs developed polymorphic VPCs of left ventricular origin (Fig. 4a), and additionally, two of them developed single monomorphic VPCs of right ventricular origin (Fig. 4b).

Table 1. Mean values of ECG parameters at different stages of the experiment

	Before MI induction	During MI induction procedure	After MI induction
HR (bpm)	91.84	89.32	88.53
PQ (ms)	101.79	109.16	129.06
QRS (ms)	82.00	80.74	69.53
QTc (ms)	490.95	480.11	458.00

MI – myocardial infarction; HR – heart rate; PQ – interval from the beginning of the P wave to the beginning of the Q wave; QRS – interval from the end of the PQ interval to the end of the S wave; QTc – interval from the start of the Q wave to the end of the T wave corrected for heart rate

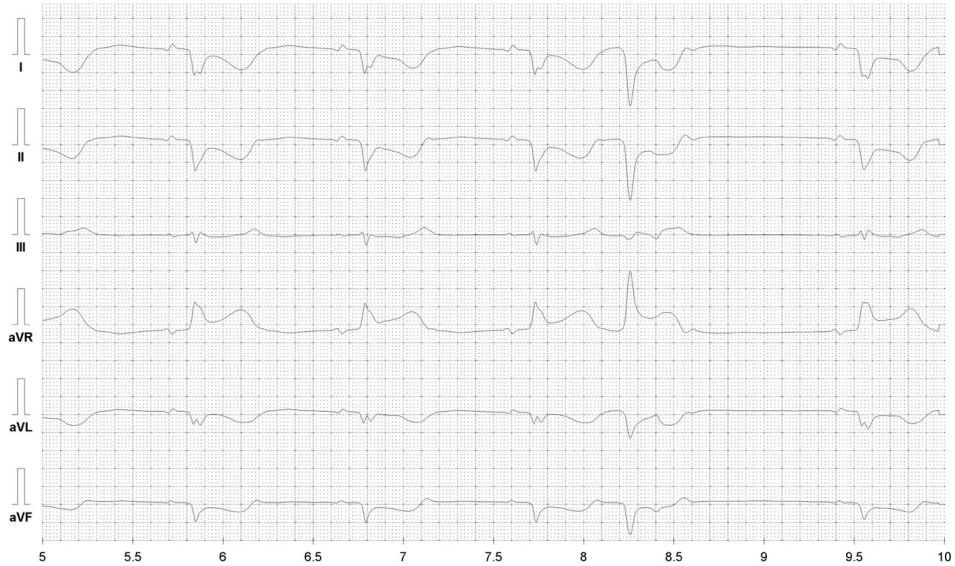

Fig. 4a. Single ventricular premature complex (the 4th beat) of left ventricular origin in a female pig subjected to myocardial infarction induced by 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol. I – bipolar limb lead, potential difference between the electrodes on the left superior limb and the right superior limb; II – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the right superior limb; III – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the left superior limb; aVR – augmented unipolar right limb lead; aVL – augmented unipolar left limb lead; aVF – augmented unipolar left hindlimb lead. The sinus rhythm is shown at a rate of 63 bpm. Paper speed: 50 mm/s; amplitude: 10 mm/mV; 25 Hz notch filter; Fuzzy+ software filter

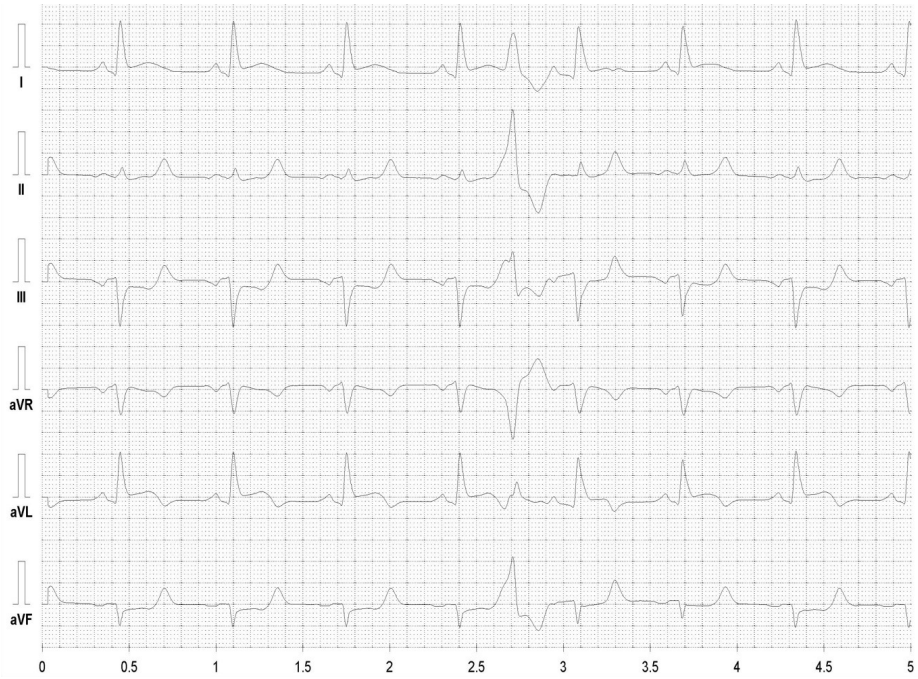

Fig. 4b. Single ventricular premature complex (the fifth beat) of right ventricular origin in a female pig subjected to myocardial infarction induced by 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol. I – bipolar limb lead, potential difference between the electrodes on the left superior limb and the right superior limb; II – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the right superior limb; III – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the left superior limb; aVR – augmented unipolar right limb lead; aVL – augmented unipolar left limb lead; aVF – augmented unipolar left hindlimb lead. The sinus rhythm is shown at a rate of 92 bpm. Paper speed: 50 mm/s; amplitude: 10 mm/mV; 25 Hz notch filter; Fuzzy+ software filter

The most common type of arrhythmia seen concurrently with VPCs was ventricular tachycardia, recorded in 11 pigs with VPCs (68.75%). Ventricular premature complexes occurred alone only in two pigs (11.11% of pigs with arrhythmias and 10.53% of all pigs). Ventricular couplets were found in 9 out of 18 pigs with arrhythmias (50% of pigs with arrhythmias and 47.37% of all pigs). Three consecutive ventricular beats (triplets) occurred in three pigs (16.67% of pigs with arrhythmias and 15.79% of all pigs). Ventricular bigeminies, defined as regular sinus beats continuously alternating with premature ventricular beats, were reported in three pigs (16.67% of pigs with arrhythmias and 15.79% of all pigs). Equally frequently occurring ventricular trigeminies (two sinus beats alternating with one premature ventricular beat) were found in three pigs (16.67% of pigs with arrhythmias and 15.79% of all pigs).

Ventricular tachycardia (Fig. 5) was diagnosed in 12 pigs (66.67% of pigs with arrhythmias and 63.16% of all pigs) and all tachycardia episodes were classified as non-sustained VT (*i.e.* lasting less than 30 s). The median duration of ventricular complexes during VT was 106 (60–246) ms and the mean duration of QRS was 80.92 ± 9.34 ms as recorded during sinus rhythm in animals before MI induction.

Three of the VT episodes were diagnosed as Torsade de Pointes (25% of pigs with VT and 16.67% of pigs with arrhythmias). The number of VT episodes recorded in animals during MI varied from 1 to 9 (Fig. 6).

Ventricular fibrillation was reported in nine animals (50% of pigs with arrhythmias and 47.37% of all pigs). Those nine pigs had 18 episodes of VF, in six of which it developed without preceding VT (33.33% of all episodes) (Fig. 7), while in 12 episodes there was a direct transition from VT to VF (66.67% of all episodes).

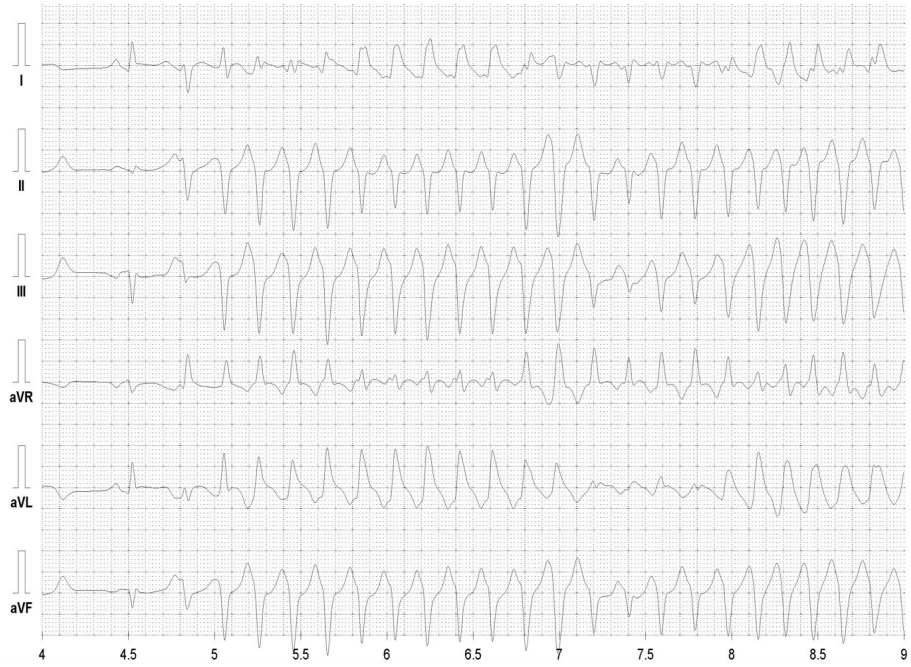

Fig. 5. Ventricular accessory R/T beat triggering ventricular tachycardia in a female pig subjected to myocardial infarction induced by 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol. I – bipolar limb lead, potential difference between the electrodes on the left superior limb and the right superior limb; II – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the right superior limb; III – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the left superior limb; aVR – augmented unipolar right limb lead; aVL – augmented unipolar left limb lead; aVF – augmented unipolar left hindlimb lead. Heart rate: 303 bpm; Paper speed: 50 mm/s; amplitude: 10 mm/mV; 25 Hz notch filter; Fuzzy+ software filter

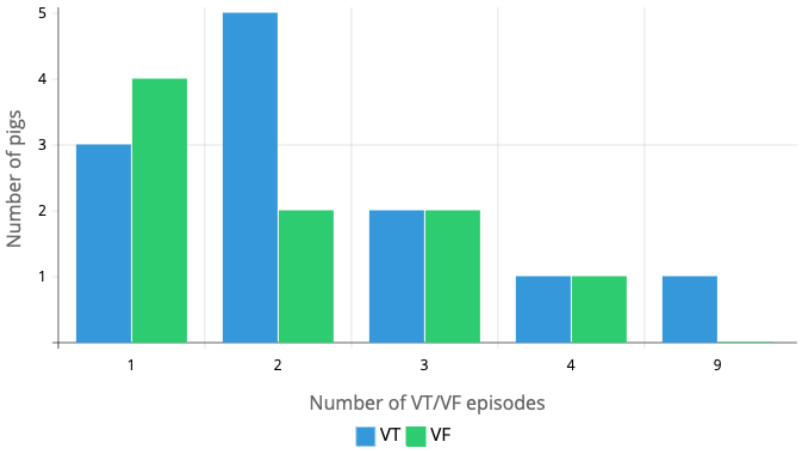

Fig. 6. The number of pigs with recorded ventricular tachycardia (VT) or ventricular fibrillation (VF) episodes during the induction of acute myocardial infarction (MI) by 30 min left anterior descending coronary artery occlusion with an angioplasty balloon in a modified anaesthetic protocol

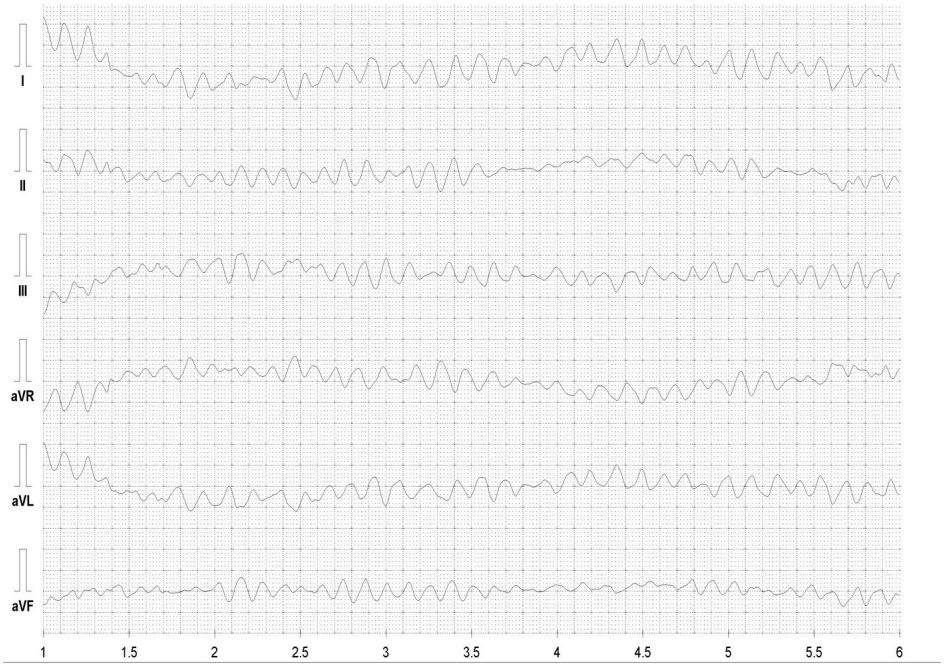

Fig. 7. Ventricular fibrillation in a female pig subjected to myocardial infarction induced by a 30 min occlusion of the proximal part of the left anterior descending coronary artery with an angioplasty balloon in a modified anaesthetic protocol. I – bipolar limb lead, potential difference between the electrodes on the left superior limb and the right superior limb; II – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the right superior limb; III – bipolar limb lead, potential difference between the electrodes on the left inferior limb and the left superior limb; aVR – augmented unipolar right limb lead; aVL – augmented unipolar left limb lead; aVF – augmented unipolar left hindlimb lead. Heart rate: 329 bpm; paper speed: 50 mm/s; amplitude: 10 mm/mV; 25 Hz notch filter; Fuzzy+ software filter

The number of VF episodes in individual pigs ranged from 1 to 4 (Fig. 6). The only arrhythmia not classified as ventricular was the first-degree atrioventricular (AV) block, diagnosed in 2 pigs (11.11% of pigs with arrhythmias and 10.53% of all pigs). The incidence of VPC was statistically significantly higher than the incidence of triplets, bigeminies, trigeminies, and the first-degree AV block ($P < 0.05$).

Coexistence of different types of arrhythmias. In pigs with cardiac arrhythmias, co-occurrence of three different types was most commonly diagnosed (6 pigs, 33.33% of pigs with arrhythmias, and 31.58% of all pigs). Simultaneous affliction with two types of arrhythmia was described in four pigs (22.22% of pigs with arrhythmias and 21.05% of all pigs). A single type of cardiac arrhythmia was found in three pigs (16.67% of pigs with arrhythmias and 15.79% of all pigs). Six different arrhythmias were reported in two pigs (11.11% of pigs with arrhythmias and 10.53% of all pigs). In contrast, multiplicities of 4, 5, or 7 different arrhythmias were diagnosed in one animal each (5.56% pigs with arrhythmias and 5.26% of all pigs).

Time of the first arrhythmia's occurrence. The first arrhythmia developed within the first 10 min of LAD occlusion in 15 (83.33%) pigs with arrhythmias, while in three pigs (16.67%) it did so between the 20th and 30th minute of LAD occlusion (median 4.5 (1–30) min). The appearance of the first VPC was also associated with two time periods. In 14 pigs with VPC (87.5%), it occurred during the first 10 minutes of ischaemia, and in two pigs with VPC (12.5%), it manifested between the 20th and 30th min of LAD occlusion (median 4.5 (1–30) min). Additionally, VPC was the first reported arrhythmia in 83.33% of pigs (n = 15) with arrhythmias. The first episodes of VT were recognised: in seven affected pigs within the first 10 min of LAD occlusion (58.33%), in two pigs between the 10th and 20th min (16.67%), and in three pigs between the 20th and 30th min (25%) (mean 13.25 ± 9.64 min). The first episodes of VF were recorded in one pig (11.11%) in the first 10 minutes, in three pigs (33.33%) between the 10th and 20th min, and in five pigs (55.56%) between the 20th and 30th min of LAD occlusion (mean 20.11 ± 6.68 min) (Fig. 8).

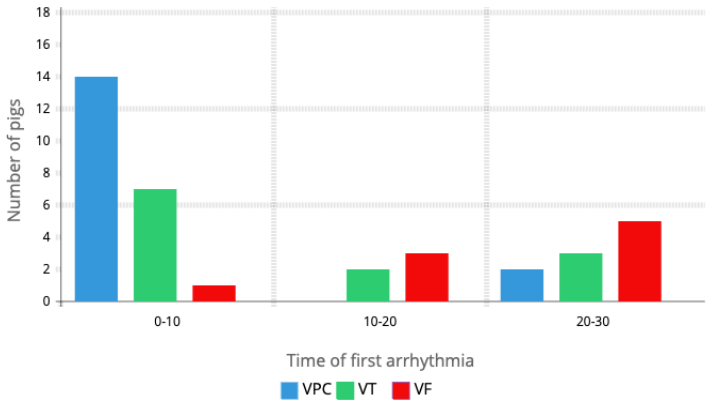

Fig. 8. The number of pigs with ventricular premature complexes (VPCs), ventricular tachycardia (VT), or ventricular fibrillation (VF) occurring for the first time within a specific time interval during induction of acute myocardial infarction by 30 min left anterior descending coronary artery occlusion with an angioplasty balloon in a modified anaesthetic protocol

Discussion

Our study aimed to characterise ventricular arrhythmias in a porcine MI model obtained by occluding the proximal part of the LAD. We used a modified protocol of animal anaesthesia consisting of a changed premedication scheme and appropriate management during the haemodynamic disorder's development, and we aimed to obtain a stable MI model with a high survival rate. In the changed premedication regimen, we used a combination of drugs from three different groups (ketamine, medetomidine and midazolam). This allowed us to use lower doses of drugs, reduce the risk of side effects characteristic for higher doses of drugs (synergism) and introduce appropriate anaesthetic management during haemodynamic stabilisation of subjects at the time of pressure drop, hypovolaemia, or shock. So far, the procedure of haemodynamic disorder correction has not been described in the literature for similar animal models. A combination of drugs from three different groups was described for premedication in pigs (47). However, the application of the substances used in our experiment was not previously reported.

In the conducted experiment, ventricular arrhythmias occurred in 94.74% of pigs subjected to myocardial ischaemia induction. The most common arrhythmia observed in our study was the VPC (88.89% of pigs with diagnosed arrhythmia), the occurrence of which was related to the time of ischaemia induction, as also reported by other studies (20, 50).

In our study, VT and VF, which may be fatal complications of acute MI, occurred in 66.67% and 50% of pigs with cardiac arrhythmias, respectively. The registered VT episodes were characterised as non-sustained and polymorphic. In some studies, the incidence of VF in pigs during LAD occlusion ranged from 50 to 75%. Other studies reported even up to 100% VF incidence (3, 4, 6, 12, 23, 26, 28, 37, 42, 52, 56). Therefore, the VF incidence rate of 50% obtained in our experiment is consistent with the previously published literature data. In this study, 83.33% of pigs with arrhythmias (15 out of 18) developed complexes in the form of two or more types of arrhythmia, which may be related to the extent of the ischaemic area created by the severely injurious LAD occlusion. In most of the studied animals, the first arrhythmias following the myocardial ischaemia induction developed within the first 10 min (83.33%). The first episodes of VPC or VT occurred in the initial 10 min of MI (87.5% and 58.33%, respectively). The second period in which a significant proportion of cardiac arrhythmias was recorded was within the last 10 min of the procedure (in 12.5% of animals VPC presented and in 25% VT). This distribution of arrhythmias over time is consistent with that noted in other studies (4, 7, 47). However, the VF reported during our study had a different distribution, as most episodes occurred at the end of ischaemia between the 20th and 30th min (55.56%).

Our experiment showed a 100% survival rate, which is very high compared to other studies on MI induced by the proximal LAD occlusion (35, 52) reporting that mortality of pigs in the MI group could reach 100%. In pig models using angioplasty balloons, acute myocardial ischaemia was usually achieved by LAD (9, 25, 26, 39, 45, 47, 52, 55, 61) or left coronary artery circumflex (LCX) balloon occlusion (44, 61, 62). Most often, LAD closure was performed in its central

part (9, 25, 28, 55, 57), mainly distally to the first septal branch (39) or diagonal branch (30, 44, 45, 48, 52, 58). The place where the LCX was closed was usually at its beginning (45, 58) or more distally (51). The mortality associated with the development of myocardial ischaemia reported in these studies ranged from 20.51% to 33%. In these studies, the LAD lumen occlusion was not performed in its proximal part, most probably because it results in a larger area of induced ischaemia. Larger ischaemia may potentially affect the course of the procedure and undoubtedly decreases the animal survival rate because of episodes of VF and haemodynamic disorders (28, 35, 52). In their study, Suzuki *et al.* (52) found that LAD occlusion in the proximal part resulted in higher animal mortality than mid-LAD occlusion. Moreover, they proved that LAD proximal lumen occlusion led to the greatest myocardial necrosis (52). Also, the mortality rate for the proximal LAD occlusion group was as high as 50%, while in the mid-LAD occlusion group, no deaths occurred (52). In another study, Munz *et al.* (35) permanently ligated the proximal LCX or LAD in three different sections, namely proximal, medial, and distal, to achieve MI (35). In the group of animals with the proximal LAD occlusion, the mortality was 100%. The authors established that the optimal place of LAD occlusion to induce MI in a pig model is its middle part (35). The effects of LAD closure location on MI development, the size of the myocardial ischaemia area, and the development of arrhythmias were confirmed by Li *et al.* (28). In this experiment, the LAD was closed in its middle and bottom-third parts. The results indicated that LAD occlusion in its middle part promotes more frequent VF and more serious haemodynamic disturbances (28). The high survival rate of the animals in our MI model could have been influenced by timely management of the disturbances caused by arrhythmias and myocardial dysfunction caused by MI. However, it may also result from using only young and healthy pigs in the presented study. A relatively homogeneous group of young and healthy pigs may be considered the main limitation of the presented study, since they do not constitute the best material to be analogous to people suffering from acute MI in terms of health and age.

Another limitation is the sex of the animals used. Mortality from coronary events in women at younger age is lower than in men of the same age group, although the differences become less pronounced in older age groups (34). The INTERHEART study showed that the first MI in women occurred on average 9 years later than in men (1). Although women tend to be less frequently affected by MI, clinical evidence suggests that they have higher mortality and a worse prognosis after an acute cardiovascular event (16).

The choice to use juvenile females in our study is related to our previous experience using a porcine model. According to our observations, females are more resistant to the stress of new housing conditions and perioperative time. Moreover, they show less aggression towards other animals and staff and become accustomed to human investigators faster than males. In addition, the use of young animals is also due to the need for venepuncture to perform the test and for effective defibrillation if VF develops. These activities in adult males are significantly hampered by their size and large body mass. Thus, experimentation on young females by choice was intended to reduce the impact of stress associated with daily maintenance and handling and the perioperative period and to facilitate the efficient performance of the planned procedures.

Based on the cited literature data, we may conclude that LAD occlusion in the proximal segment is not optimal for establishing a porcine MI model. The procedure results in high mortality of the animals, which makes it impossible to monitor a sufficient number of them or assess the effects of LAD closures over the long term. However, it is essential to develop a stable model for MI induction *via* proximal LAD occlusion conducive to research on arrhythmias and their outcomes and prognosis, which is vital for people who develop MI because of proximal LAD occlusion. Such a model would enable researchers to better understand the pathophysiological changes in the myocardium due to occlusion in the LAD proximal segment. The anaesthetic protocol used in our study (consisting of haemodynamic stabilisation of patients at the time of pressure drop, hypovolaemia or shock) allowed for the effective treatment of life-threatening haemodynamic disorders and arrhythmias caused by myocardial ischaemia and for the correction of the condition of animals with already developing hypotension, hypovolaemia, or shock. Thanks to haemodynamic stabilisation, we achieved 100% survival of the animals subjected to the ischaemia induction and reperfusion procedure.

In conclusion, myocardial ischaemia obtained by LAD proximal segment occlusion is characterised by a high rate of cardiac arrhythmias. The most frequently recorded cardiac arrhythmias were ventricular tachycardia and ventricular fibrillation, which usually pose a direct threat to life. The applied modified anaesthesia management, aiming to stabilise haemodynamic disorders caused by myocardial infarction and arrhythmias, ensured 100% ischaemia and reperfusion survival. Such results qualify the described MI model as worthwhile to adopt for research on arrhythmias in MI due to proximal LAD occlusion in humans. The proposed protocol significantly affects the survival of animals in the presented MI model, which enhances its potential for use in the study of ventricular arrhythmias in acute myocardial ischaemia.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The study was carried out according to the guidelines of the National Institute of Health for the care and use of laboratory animals. All the experimental protocols involving animals were approved by the Local Ethical Committee for Animal Experiments at the Wrocław University of Environmental and Life Sciences (Resolution no. 081/2019, 11.12.2019).

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Cardioprotective Effect of Acetylsalicylic Acid in the Myocardial Ischemia-Reperfusion Model on Oxidative Stress Markers Levels in Heart Muscle and Serum

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Abstract: Heart failure occurs in increased oxidative stress conditions, which contribute to the progression of pathological changes. Orally or intravenously administered acetylsalicylic acid (ASA, aspirin) is typically used in human patients with acute myocardial ischemia. The study used an experimental porcine ischemia-reperfusion model to evaluate the potential cardioprotective effect of intracoronary administered ASA on myocardial ischemia-reperfusion injury. The cardioprotective effect of ASA was evaluated by measuring selected oxidative stress markers levels in infarcted and non-infarcted myocardium 14 days after the procedure, and three times in serum, before the procedure, during the reperfusion process, and after 14-day recovery. The results showed that intracoronary administrated ASA reduced the oxidative stress. The level of oxidative stress, measured with the non-enzymatic markers total antioxidant capacity (TAC), total oxidative status (TOS), and malondialdehyde (MDA), and the enzymatic markers glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST), in heart tissue was significantly higher in a control group injected with saline. The level of oxidative stress in serum, measured with TAC, TOS, oxidative stress index (OSI), and lipofuscin (LF), was also higher in the control group than in animals injected with ASA. The confirmed cardioprotective effect of intracoronary administered ASA provides the foundation for further studies on ASA intracoronary application, which may lead to the development of a new therapy for the treatment of ischemia-reperfusion complications in humans.

Keywords: myocardial ischemia-reperfusion; acetylsalicylic acid; oxidative stress markers

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1. Introduction

Cardiovascular diseases (CVDs) and their complications are the most common causes of death in industrialized countries [1]. Over the past several decades, investigations into human heart failure and animal models of heart failure have provided substantial evidence that oxidative stress increases during heart failure and contributes to disease progression [2]. Oxidative stress (OS) is described as the disturbance in the equilibrium of pro- and antioxidants in favor of the prooxidants. Oxidative stress contributes significantly to the atherogenic processes [3,4]. In isolated heart cells, it changes the gene expression

and induces cell death that accompanies myocardial remodeling and heart failure [2]. Hyperlipidemia, hypertension, heart diseases, and other conditions linked to CVD are associated with prooxidant overproduction or endogenous antioxidant deficiencies [5–7]. Prooxidants comprise free radical species or non-radical species mediating peroxidation and include, among others, reactive oxygen species (ROS) and reactive nitrogen species (RNS) [4]. In acute myocardial infarction (MI), reactive oxygen species (ROS) are generated in the ischemic myocardium, especially after reperfusion. Reactive oxygen species directly injure the cell membrane and cause cell death [8]. Myocardial infarction and heart failure are CVDs considered valid research targets for prooxidant–antioxidant imbalance [4]. The therapeutic effects of antioxidants on heart failure progression have already been reported [2].

Unfortunately, the mechanism of myocardial damage caused by ischemia and subsequent reperfusion (IR) is yet to be fully understood, but inflammation and platelet activation to post-reperfusion injury play a significant role [9,10]. Aspirin (acetylsalicylic acid, ASA) is an anti-inflammatory and antiplatelet drug administered orally to patients with the acute coronary syndrome. Its protective role results from inhibiting cyclooxygenases (COXs) that metabolize arachidonic acid and produce prostaglandin. Although ASA does not affect cardiovascular function directly, it seems to effectively protect against many cardiovascular pathologies such as atherosclerosis, ischemic heart disease, and myocardial infarction. Intracoronary ASA administration during acute ischemia was hoped to reduce the inflammatory response and, in consequence, block platelets [11]. Studies showed that intravenous ASA administration did not reduce the size of post-ischemia necrosis. However, it inhibited the conversion of PGG and PGH prostaglandins to thromboxane A2, and their further conversion to the cardioprotective PGE, PGD, and PGI2 prostaglandins, by blocking platelet cyclooxygenase [12]. Other studies showed that ASA protects the heart muscle against damage caused by energy metabolism disturbances occurring during an ischemic episode. The cardioprotective effect results from blocking the production of prostaglandins and stabilizing the cell membrane [11]. Other studies have showed that ASA inhibits prostacyclin production in heart tissue by blocking COX, which exacerbates myocardial dysfunction induced by IR episode [12]. Studies on intracoronary drug administration and the effectiveness of intravenous vs. intracoronary drug administration have showed that anticoagulants (GP IIb/IIIa receptor inhibitors) are much more effective when administered via an intracoronary route. They reduce thrombus growth and improve coronary blood flow [13,14]. Intracoronary administration of abciximab (an antiplatelet drug, the glycoprotein IIb/IIIa inhibitor) improved myocardial reperfusion, as reflected by a decrease in enzymatic markers in patients with myocardial infarction [15]. It can be assumed that intracoronary ASA administration may more effectively improve the coronary blood flow in infarction compared to oral or intravenous (peripheral) administration.

In the past decade, various animal models of human diseases have been developed for cardiovascular research on myocardial regeneration and therapy [16,17]. One of the most commonly used models is the porcine model of ischemia-reperfusion [18]. The swine (*Sus scrofa domestica*) heart is anatomically similar to the human heart. Lesions induced by ischemia-reperfusion in the porcine model represent well the injury occurring in patients who have suffered from an acute myocardial infarction. The standard treatment procedure in myocardial ischemia is the restoration of blood flow in the coronary vessels (PCI procedure) [19]. However, the risk of damage due to reperfusion after previous ischemia is considerable [20].

The present study investigated the potential cardioprotective effects of intracoronary administered aspirin (ASA) on the ischemia-reperfusion model using selected oxidative stress markers. To fulfil the goal, we studied levels of non-enzymatic and enzymatic oxidative stress markers in serum and heart muscle tissue collected from infarcted and non-infarcted parts of the left ventricle of the heart. Confirming the cardioprotective effect of aspirin administered to the coronary vascular system in the porcine model would provide the basis for proposing this new pharmacological strategy to clinicians. The results from

our study would help to expand the prevention and treatment methods for heart damage after coronary arteries ischemia and reperfusion episodes in human patients.

2. Materials and Methods

2.1. Ethical Statement and Permissions, Animals

The research complied with the National Institute of Health guidelines for the care and use of laboratory animals. The experimental protocol was approved by the Local Ethics Committee for Animal Experiments at Wroclaw University of Environmental and Life Sciences, Poland (protocol no. 081/2019, approved on 11 December 2019).

Female pigs (*Sus scrofa domestica*) of the Polska Biała Zwisłoucha breed (n = 13, 16–20 weeks old, 33–44 kg) were purchased from the Experimental Station of the National Research Institute of Animal Production in Żerniki Wielkie (Poland). The use of female pigs stems from our previous studies in a porcine model. Female pigs exhibit greater resilience to stress associated with daily handling, grooming, and new housing conditions. In addition, they are more resistant to perioperative stress. Females are also less aggressive towards other animals and staff and quickly habituate to animal handling staff.

The animals were housed and maintained in the same controlled conditions, with a diet conforming to nutritional standards and water ad libitum. All animals were habituated to grooming activities before the study started.

2.2. Study Groups

Animals were divided into the following two groups: control group (n = 6) and ASA group (n = 7). Animals from both groups were subjected to the acute myocardial ischemia protocol. During the reperfusion period, after inducing myocardial ischemia, the animals from the control group received an intracoronary injection of NaCl, while animals from the ASA group received an intracoronary injection of acetylsalicylic acid. All animals subjected to the experiment survived the induction the myocardial ischemia, the reperfusion period, and the 14-day recovery time (100% survival rate).

Evaluation of the Animals' Health Status

The pigs' health status before the induction of myocardial ischemia, during the reperfusion period, and after the 14-day recovery time (before euthanasia) was evaluated based on clinical examination and biochemical (aspartate aminotransferase (AST), urea, creatinine, and fibrinogen levels) and morphological blood tests (white blood cells (WBC) and red blood cells (RBC) count).

2.3. Protocol for Inducing Acute Myocardial Ischemia in a Porcine Model

2.3.1. Premedication

The animals fasted for 12 h before the procedures started. Premedication was performed with intramuscular injection of 10 mg/kg b.m. of ketamine (Vetaketam 100 mg/mL, Vet-Agro Sp. z.o.o., Lublin, Poland), 0.3 mg/kg b.m. of midazolam (Midanium 5 mg/mL, Polfa Warszawa S.A., Warsaw, Poland), and 0.03 mg/kg b.m. of medetomidine (Sedator 1 mg/mL, Eurovet Animal Health BV, Bladel, The Netherlands).

2.3.2. General Anesthesia Induction and Maintenance

After immobilization, the animals were placed in a sternal lying posture, and the marginal vein of the ear was catheterized in order to obtain vascular access. General anesthesia was induced with an intravenous bolus (2 mg/kg b.m.) of propofol (Provive 10 mg/mL, Claris Lifesciences UK Ltd., Crewe, Cheshire, UK). Then, the animals were intubated with a size 8 endotracheal tube (Tracheal Tube type Murphy, SUMI, Sulejówek, Poland) and mechanically ventilated for the remainder of the procedure using 100% oxygen and a closed gas system with a carbon dioxide absorber. The initial phase of ventilation was pressure-controlled, and after evaluating the ventilation parameters and capnometry, the ventilation switched to volume-controlled mode. The oxygen flow was maintained at

2 L/min, with a tidal volume of 10 mL/kg b.m. and respiratory rate of 12/min. Ventilator (Primus, Dräger Medical AG & Co. KGaA, Lübeck, Germany) settings were adjusted individually to obtain end-tidal CO_2 concentration between 35 and 45 mmHg.

General anesthesia was maintained with isoflurane (Forane, Abbott Laboratories, Warsaw, Poland) inhaled via a calibrated vaporizer (Vapor 2000, Dräger Medical AG & Co. KGaA, Lübeck, Germany). The concentration of isoflurane (1.5–2.5%) in 100% oxygen was monitored (Lifepak 12, Medtronic, Redmond, WA, USA) and adjusted according to the capnometry value, to achieve 1 MAC (minimum alveolar concentration). Analgesia was achieved by injecting an intravenous bolus (10 µg/kg b.m.) of fentanyl (Fentanyl WZF 50 µg/mL, Polfa Warszawa S.A., Warsaw, Poland) and continued with its intravenous infusion at 10 µg/kg b.m./h rate.

2.3.3. Hemodynamic Stability Maintenance

In the intra- and perioperative period, fluid therapy was performed using constant rate infusion (CRI) of a multi-electrolyte solution (Optilyte, Fresenius Kabi Polska Sp. z o.o., Kutno, Poland) at 6–12 mL/kg b.m./h rate, based on the animal's hydration status and response to medication.

During the procedure, the following vital functions of the animals were monitored (Lifepak 12, Medtronic, Redmond, WA, USA): internal body temperature, saturation, pulse, and blood pressure. Additionally, the heart rhythm was constantly tracked using a 12-lead ECG (BTL-08 MT Plus ECG, BTL Industries Ltd., Stevenage, Herefordshire, UK).

Hypotension events occurring during the procedure were stabilized with 10 mL/kg b.m boluses of lactated Ringer's solution (Solutio Ringeri Lactate Fresenius, Fresenius Kabi Polska Sp. z o.o., Kutno, Poland) and 3–5 mL/kg b.m. boluses of hydroxyethyl starch 130/0.4 (HES; Voluven, Fresenius Kabi Deutschland GmBH, Bad Homburg vor der Höhe, Germany). In the absence of adequate response of the circulatory system to fluid resuscitation, continuous intravenous infusion of dopamine (Dopaminum Hydrochloricum WZF 40 mg/mL, Polfa Warszawa S.A., Warsaw, Poland) was administered at an initial dose of 4 µg/kg b.m./min, and afterwards, the dosage was adjusted to the animal's clinical status and vital parameters. Additionally, the Trendelenburg position was periodically employed to rebalance the circulatory system function.

2.3.4. Myocardial Ischemia Induction

Acute myocardial ischemia was obtained following previously described protocols [21–23] with minor modifications.

Percutaneous vascular access, via the vascular sheath (6F diameter, Balton, Warsaw, Poland), to the femoral artery was gained using a puncture needle (21G, Balton, Warsaw, Poland) and ultrasound guidance (F37, Hitachi Aloka Medical Ltd., Mure, Mitaka-shi, Tokyo, Japan). After inserting the guide catheter (Launcher, JL 3.5 curvatures, 6F diameter, Medtronic, Santa Rosa, CA, USA), a 6000 UI heparin bolus (Heparinum WZF 5000 UI/mL, Polfa Warszawa S.A., Warsaw, Poland) was injected, and the coronary arteries were assessed by angiography.

Afterwards, an angioplasty guidewire (BMW, 3 m, 0.014", Abbott, Santa Clara, CA, USA) was introduced through the catheter and positioned in the proximal segment of the left anterior descending artery (LAD) under the control of fluoroscopy (Symbol, General Medical Merate SpA, Seriate, Italy). A 3.0 × 10 mm angioplasty balloon (Sprinter, OTW model, Medtronic, Santa Rosa, CA, USA) was located on the guidewire and inflated to a pressure of 6 atm for 30 min to obtain complete LAD closure. The occlusion of the LAD was verified by angiography, and myocardial infarction was recognized by ST-segment elevation on the 12-lead ECG.

2.3.5. Reperfusion and Acetylsalicylic Acid (ASA) Treatment

Reperfusion was achieved by deflating the angioplasty balloon and restoring blood supply to the ischemic part of the myocardium. Ten minutes before emptying the balloon,

animals belonging to the ASA group were injected intracoronary with 150 mg of acetylsalicylic acid (Kardegic 0.5 g 500 mg/5 mL, Sanofi-Aventis s.r.o., Praha, Czech Republic), whereas animals in the control group were injected intracoronary with the same volume of 0.9% NaCl. The acetylsalicylic acid dose was determined based on recommendations regarding its use in patients with acute coronary syndrome [24]. The invasive procedure of myocardial infarction induction and reperfusion was concluded 15 min after deflating the angioplasty balloon.

2.4. Postoperative Treatment and the Recovery Time

Postoperatively, an adequate analgesic treatment was provided by intramuscular injections of buprenorphine (Bupaq Multidose 0.3 mg/mL, Richter Pharma AG, Wels, Austria) (0.01 mg/kg b.m., every 8 h) for 3 days, metamizole (Pyralgivet 500 mg/mL, Vet-Agro Sp. Z.o.o., Lublin, Poland) (50 mg/kg b.m., every 24 h) for 3 days, and meloxicam (Metacam 5 mg/mL Boehringer Ingelheim Vetmedica GmBH, Ingelheim/Rhein, Germany) (0.4 mg/kg b.m., every 24 h) for 2 days. Afterwards, until the 14th day after the procedure, the animals were housed and maintained as before the experiment.

2.5. Blood Sample Collection

Blood for biochemical and morphological analyses was collected in plastic tubes with a clot activator or EDTA, respectively. The first sample was collected shortly after cannulating the right femoral artery before inducing ischemia. The second sample was collected during the reperfusion period, 15 min after deflating the angioplasty balloon and 25 min after ASA administration. The third sample was collected before the animal was euthanized. Blood for morphological analyses was collected before inducing ischemia and before euthanizing the animals.

2.6. Euthanasia and Heart Tissue Collection

The animals were euthanized 14 days after acute myocardial ischemia induction. The animals were premedicated according to the protocol described above (intramuscular administration of ketamine, midazolam, and medetomidine). Then, propofol was administered through the marginal vein of the ear. After inducing anesthesia, the third blood sample for biochemical analyses was collected through the right femoral artery. Euthanasia was performed by an intravenous administration of a 100 mg/kg b.m.of pentobarbital and sodium pentobarbital mixture (Morbital 133.3 mg/mL + 26.7 mg/mL, Biowet Puławy Sp z o.o., Puławy, Poland).

After completing the euthanasia procedure, each animal from the control and from the ASA group was subjected to an autopsy, during which two samples (100 mg each) of the left ventricular myocardium were collected, one from the infarcted (the necrotic area induced by the ischemia) and one from the non-infarcted area (visible healthy tissue) of the left ventricular myocardium. The cardiac tissue was collected to 1 mL of a homogenizing buffer with protease inhibitors. Then, it was homogenized (1:10 *w/v*) in 0.9% NaCl with a glass homogenizer (Potter-Elvehjem PTFE, Sigma-Aldrich, Darmstadt, Germany) and sonicated (Virsonic 100, VirTis, Gardiner, NY, USA). The lysate was centrifuged for 10 min, at 4000× *g* rpm, at 4 °C, and treated as one independent sample. The tissue samples were then frozen and stored at −80 °C until the analysis.

2.7. Oxidative Stress Marker Analysis

Oxidative stress markers were analyzed in the left ventricle samples (infarcted and non-infarcted) and in the blood serum samples. In the left ventricle samples, the following non-enzymatic oxidative stress markers were analyzed: total antioxidant capacity (TAC), total oxidative status (TOS), oxidative stress index (OSI), and malondialdehyde (MDA) concentration, and activity of the enzymatic oxidative stress markers glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST). Concentrations

of the non-enzymatic oxidative stress markers TAC, TOS, OSI, and lipofuscin (LF) were analyzed in the serum samples.

2.7.1. Total Antioxidant Capacity

Total antioxidant capacity (TAC) was assessed using a Randox TAS assay kit (Randox Co., Crumlin, County Antrim, UK). ABTS (2,2′ azino-di-(3-ethylbenzothiazoline sulphonate) was incubated with a peroxidase (metmyoglobin), hydrogen peroxide, and the sample to obtain the radical cation (ABTS+), in which a blue-green color can be measured at 600 nm. Suppression of the color was compared to the standard for TAC measurement assays (Trolox), and the results were expressed as a Trolox equivalent (mmol/L) [25].

2.7.2. Total Oxidative Status

Total oxidative status (TOS) was determined as described by Erel [25]. The method uses the oxidation of Fe^{2+} to Fe^{3+} in an acidic medium. The produced Fe^{3+} ions react with xylene orange and form a colorful blue-purple complex, detected at 560 nm. The TOS level was determined against the calibration curve using H_2O_2 as the standard, and the results were expressed in μmol/L.

2.7.3. Oxidative Stress Index

Oxidative stress index (OSI) was expressed as the total oxidant status to total antioxidant capacity (TOS/TAC) ratio and expressed in arbitrary units [26].

2.7.4. Malondialdehyde Concentration

Malondialdehyde (MDA) concentration in samples was determined using the method of Ohkawa et al. with thiobarbituric acid. MDA concentration was detected spectrophotometrically (at 515 nm for excitation and at 552 nm for emission), calculated from the standard curve prepared using 1,1,3,3-tetraethoxypropane and expressed in μmol/L [27].

2.7.5. Glutathione Peroxidase Activity (EC 1.11.1.9)

Glutathione peroxidase (GPx) activity was assessed using the kinetic method described by Mannervik. The decrease in NADPH concentration was monitored spectrophotometrically at 340 nm for 10 min, and GPx activity was expressed in IU/mg protein [28].

2.7.6. Glutathione Reductase Activity (EC 1.8.1.7)

Glutathione reductase (GR) activity was assessed using the kinetic method described by Carlberg and Mannervik. The decrease in NADPH concentration in the samples was monitored at 340 nm for 10 min, and GR activity was expressed in IU/mg protein [29].

2.7.7. Glutathione S-Transferase Activity (EC 2.5.1.18)

Glutathione S-transferase (GST) activity was evaluated using the kinetic method described by Habig and Jakoby [30] with 1-chloro-2,3-dinitro-benzene as a reaction substrate. GST activity was expressed in IU/mg protein.

2.7.8. Lipofuscin Concentration

Lipofuscin (LF) concentration was determined as described by Tsuchida et al. [31]. The serum sample was mixed with ethanol-ether (3:1, *v/v*), shaken, and centrifuged. The fluorescence intensity was determined at 345 nm (absorbance) and 430 nm (emission). LF concentration was expressed in relative lipid extract fluorescence (RF), where 100 RF corresponds to the fluorescence of 1 μg/mL quinidine sulfate in 0.1 N sulfuric acid.

2.8. Statistical Analysis

Statistical analysis was performed using Statistica, version 13 (TIBCO Software Inc., Palo Alto, CA, USA). Statistical significance was set at $p < 0.05$. Data distribution was assessed using the Shapiro–Wilk test. The mean ± standard deviation (SD) was calculated

for normally distributed data. The median with upper and lower quartile (Me (Q_1; Q_3)) was determined for data with a skewed distribution. Data with a skewed distribution were log-transformed before further analyses. A two-way ANOVA analysis with contrast analysis was used for concentrations comparison in the heart tissues samples (oxidative stress markers) and in the blood (health status markers). An analysis of variance for repeated measures was used to compare concentrations in the serum samples (oxidative stress markers) and in the blood (health status markers), and Mauchly's test was used to assess the sphericity assumption.

3. Results

3.1. Biochemical and Morphological Blood Tests

The results of biochemical and morphological blood tests, except for fibrinogen, were within the normal ranges at each phase of the experiment (Table 1).

Table 1. Levels of biochemical and morphological blood tests used to assess the health status of pigs (n = 13) subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA). The results are presented as mean ± standard deviation and were analyzed using two-way ANOVA.

Assay	Group	Before Ischemia	During Reperfusion	Before Euthanasia	Reference Range (Min-Max)
AST [U/L]	Control (n = 6)	33.1 ± 9.0	40.5 ± 10.0	35.6 ± 9.6	16–65
	ASA (n = 7)	36.9 ± 8.9	42.3 ± 11.7	39.4 ± 22.7	
Urea [mmol/L]	Control (n = 6)	5.2 ± 1.0	6.0 ± 1.6	4.8 ± 1.7	3.3–6.6
	ASA (n = 7)	5.7 ± 1.0	6.4 ± 0.8	5.2 ± 1.0	
Creatinine [µmol/L]	Control (n = 6)	143.2 ± 40.7	165.5 ± 59.0	115.0 ± 38.3	88–239
	ASA (n = 7)	130.7 ± 27.5	155.6 ± 31.0	107.9 ± 35.3	
Fibrinogen [g/L]	Control (n = 6)	3.5 ± 0.3	2.8 ± 0.2	4.1 ± 1.2	2–4
	ASA (n = 7)	3.7 ± 0.6	2.9 ± 0.6	3.8 ± 0.4	
WBC [10^9/L]	Control (n = 6)	13.8 ± 2.9	–	14.4 ± 6.8	10–20
	ASA (n = 7)	14.7 ± 6.8	–	14.5 ± 2.1	
RBC [10^{12}/L]	Control (n = 6)	6.0 ± 0.2	–	6.0 ± 0.5	5–8
	ASA (n = 7)	6.0 ± 0.5	–	6.0 ± 0.7	

Abbreviations: ASA—acetylsalicylic acid, AST—aspartate aminotransferase, RBC—red blood cells, WBC—white blood cells.

We did not observe statistically significant differences in AST (p = 0.470), WBC (p = 0.861) and RBC (p = 0.870) concentrations between all stages of the experiment. Additionally, we did not observe statistically significant differences between control and ASA groups for these three parameters (p = 0.801 for WBC, and p = 0.964 for RBC).

We observed statistically significant differences in urea concentrations at different experiment stages ($p < 0.05$), with the highest urea concentration during the reperfusion. We found no statistically significant differences between the control and ASA group (p = 0.369), and no interaction between these two variables (p = 0.968).

We noted that creatinine levels differed significantly between the experiment stages ($p < 0.01$), but found no differences between the control and ASA group (p = 0.562) nor any interactions between variables (p = 0.977). Creatinine levels in the control group differed between before ischemia and before euthanasia ($p < 0.05$) and during reperfusion and before euthanasia ($p < 0.05$). In the ASA group, the creatinine levels differed only between the reperfusion and euthanasia stages ($p < 0.05$).

Fibrinogen levels were significantly different at each stage of the experiment ($p < 0.001$), but no differences were detected between groups of animals (p = 0.509). For individual animal groups, differences were found for fibrinogen levels before ischemia and during reperfusion ($p_{control} < 0.001$, $p_{ASA} < 0.001$) and for reperfusion and euthanasia ($p_{control} < 0.01$,

$p_{ASA} < 0.05$) stages. Fibrinogen levels in the control group before euthanasia were minimally above the reference range, while at other stages of the experiment, they were within the normal range.

3.2. Heart Muscle Tissue

We observed statistically significant differences in TAC levels between the infarcted and non-infarcted tissue of the left ventricle ($p < 0.001$). In addition, we found that TAC levels were significantly higher in the heart tissue collected from animals treated with intracoronary-administered ASA (Table 2 and Figure 1A). Moreover, the TAC level depended on the interaction between the infarction presence and drug administration. The contrast analysis showed that, regardless of the status of the collected heart tissue—infarcted or non-infarcted—TAC levels were statistically different after the administration of drugs (ASA or NaCl) (Table 3). No statistically significant differences in TAC levels were observed between the infarcted tissue of animals from the ASA and control groups. Contrast analysis showed that the TAC level in the non-infarcted tissue was higher than in the infarcted tissue (Table 3). Moreover, when considering the infarcted heart tissue only, we found differences in TAC levels between animals treated with ASA and animals from the control group ($p < 0.001$).

Table 2. Oxidative stress markers levels in infarcted and the non-infarcted tissues collected from the left ventricle of the heart of pigs ($n = 13$) subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA). The results are presented as mean ± standard deviation or median (lower, upper quartile) and were analyzed using two-way ANOVA.

Oxidative Stress Marker	Health Status of the Left Ventricle Tissue	ASA Group ($n = 7$)	Control Group ($n = 6$)	p_{tissue}	p_{drug}	$p_{interaction}$
TAC [mmol/L]	infarcted non-infarcted	6.4 ± 0.5 36.2 ± 1.9	4.8 ± 0.9 29.4 ± 2.2	<0.001	<0.001	<0.001
TOS [μmol/L]	infarcted non-infarcted	6.1 ± 0.6 4.6 ± 1.2	12.2 ± 1.4 6.4 ±0.9	<0.001	<0.001	<0.001
OSI * [index]	infarcted non-infarcted	93.7 (90.0–103.2) 11.8 (10.1–14.8)	236.7 (220.7–331.6) 21.7 (19.6–24.5)	<0.001	<0.001	<0.05
MDA [μmol/L]	infarcted non-infarcted	3.7 ± 0.3 0.8 ± 0.2	6.4 ± 0.7 1.2 ± 0.2	<0.001	<0.001	<0.001
GPx [IU/mg protein]	infarcted non-infarcted	77.9 ± 3.6 34.1 ± 2.7	96.5 ± 1.7 40.1 ± 3.0	<0.001	<0.001	<0.001
GR [IU/mg protein]	infarcted non-infarcted	3.4 ± 0.3 2.8 ± 0.5	5.6 ± 0.3 3.3 ± 0.7	<0.001	<0.001	<0.001
GST [IU/mg protein]	infarcted non-infarcted	18.4 ± 1.5 15.1 ± 0.4	18.9 ± 1.0 14.7 ± 0.4	<0.001	0.929	0.232

* Log-transformed data; Abbreviations: ASA—acetylsalicylic acid, GPx—glutathione peroxidase, GR—glutathione reductase, GST—glutathione S-transferase, MDA—malondialdehyde, OSI—oxidative stress index, TAC—total antioxidant capacity, TOS—total oxidative status.

We found statistically significant differences between TOS levels in the infarcted and the non-infarcted tissue of the left ventricle, regardless of the treatment received ($p < 0.001$). Higher TOS levels were observed in the infarcted heart tissue (Table 2, Figure 1B). Also, we found statistical differences in TOS levels between the ASA group and the control group ($p < 0.001$) when not taking the status of the collected tissues (infarcted vs. non-infarcted) into consideration. TOS levels depended on the interaction between the two analyzed parameters. Contrast analysis showed that myocardial infarction increased TOS levels. TOS levels in the infarcted tissue were higher than in the non-infarcted tissue and depended on the treatment the animals received ($p < 0.001$ for the control group and $p < 0.05$ for the ASA

group). When analyzing the myocardial tissue status individually, we found that, for the non-infarcted tissue, TOS levels were higher in the control group, while for the infarcted tissue they were higher in the ASA group.

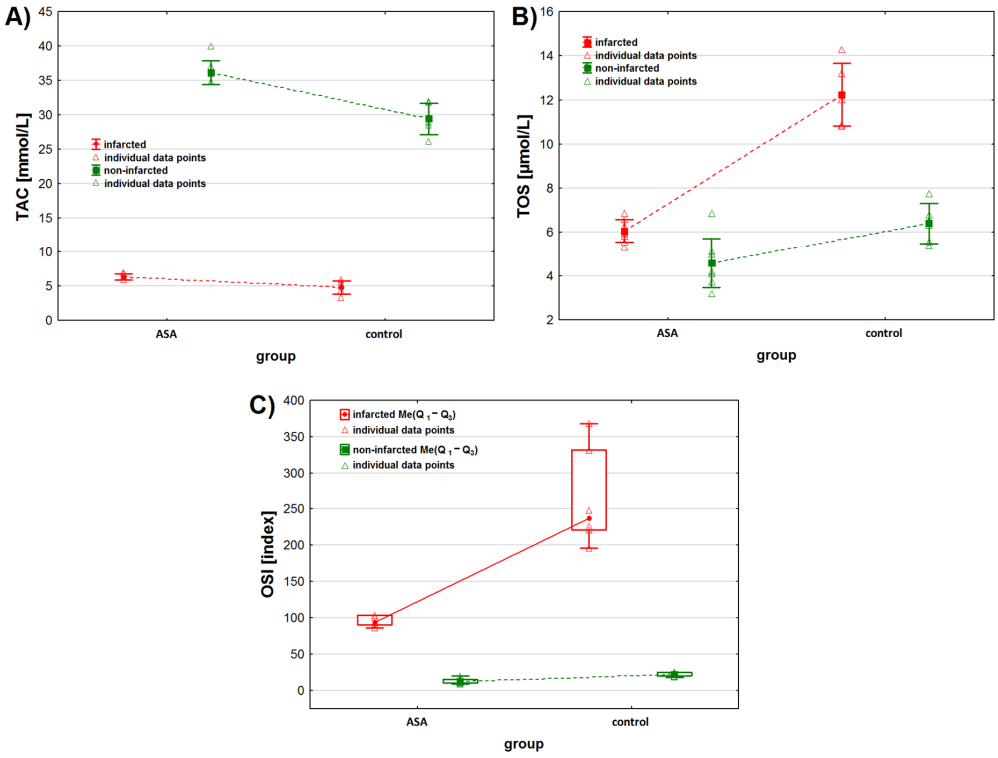

Figure 1. Oxidative stress markers levels in infarcted and the non-infarcted tissues collected from the left ventricle of the heart of pigs (n = 13) subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA): (**A**) total antioxidant capacity (TAC) [mmol/L], (**B**) total oxidative status (TOS) [µmol/L] and (**C**) oxidative stress index (OSI) [index]. Results in (**A**,**B**) are presented as mean ± 95% confidence interval and in (**C**) as median (lower, upper quartile) and minimum and maximum. In all figures, triangular markers represent the raw data. For the reader's convenience, the points are connected with dashed lines.

We found statistically significant differences in OSI values between the infarcted and non-infarcted tissue, regardless of ASA or saline use ($p < 0.001$). Higher OSI values were noted for the infarcted heart tissue (Table 2, Figure 1C). We found statistically significant differences in OSI values between the ASA and control group, regardless of the status of the collected heart ($p < 0.001$). The highest OSI values were observed in the control group. OSI values also depended on the interaction between the analyzed factors ($p < 0.05$). Contrast analysis showed that myocardial infarction significantly increased OSI values. OSI values in the infarcted tissue were higher than in non-infarcted tissue and depended on the treatment the animals received ($p < 0.001$, respectively). We found higher OSI values in the non-infarcted tissue of the control group.

Table 3. Contrast analysis of oxidative stress markers in infarcted and the non-infarcted tissues collected from the left ventricle of the heart of pigs (n = 13) subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA).

Oxidative Stress Markers	Health Status of the Left Ventricle Tissue	$P_{ASA\ vs.\ control}$	$P_{infarcted\ vs.\ non\text{-}infarcted}$ in ASA Group	$P_{infarcted\ vs.\ non\text{-}infarcted}$ in Control Group
TAC [mmol/L]	infarcted non-infarcted	0.770 <0.001	<0.001	<0.001
TOS [µmol/L]	infarcted non-infarcted	<0.01 <0.001	<0.05	<0.001
OSI * [index]	infarcted non-infarcted	<0.001 <0.001	<0.001	<0.001
MDA [µmol/L]	infarcted non-infarcted	<0.001 0.092	<0.001	<0.001
GPx [IU/mg protein]	infarcted non-infarcted	<0.001 <0.01	<0.001	<0.001
GR [IU/mg protein]	infarcted non-infarcted	<0.001 0.075	<0.05	<0.001
GST [IU/mg protein]	infarcted non-infarcted	0.360 0.429	<0.001	<0.001

* Log-transformed data; Abbreviations: ASA—acetylsalicylic acid, GPx—glutathione peroxidase, GR—glutathione reductase, GST—glutathione S-transferase, MDA—malondialdehyde, OSI—oxidative stress index, TAC—total antioxidant capacity, TOS—total oxidative status.

As for MDA, we found higher MDA concentrations in the non-infarcted tissue when considering the status of the tissue only ($p < 0.001$) (Table 2, Figure 2A). Similarly, when considering the animals' treatment only, we found higher MDA concentrations in the tissues collected from animals from the control group ($p < 0.001$). However, the interaction between both tested factors was also found to be statistically significant. Contrast analysis showed that MDA concentration was higher in the infarcted than in the non-infarcted heart tissue of animals from both study groups ($p < 0.001$ for both groups). The analysis of the non-infarcted tissue showed no difference in MDA concentration between animals injected with ASA and NaCl ($p = 0.092$), but higher MDA concentrations were observed in the infarcted heart tissue sampled from control group ($p < 0.001$) (Tables 2 and 3).

As for GPx activity, we found higher GPx activity in the infarcted tissue compared to the non-infarcted tissue and in the heart tissue collected from the control animals compared to the ASA treated animals (Table 2, Figure 2B) when considering these two factors separately (tissue status and treatment type). However, the interaction between the tissue status and the treatment type was also found to be significant ($p < 0.001$). Contrast analysis showed the same pattern for data distribution when considering both factors together. GPx activity was higher in the infarcted tissue and in animals from the control group (Tables 2 and 3).

We noted higher GR activity in the infarcted tissue than in the non-infarcted tissue ($p < 0.001$), when considering the tissue status only (Table 2, Figure 2C). No statistical differences in GR activity were noted between the ASA and the control group, when considering the type of treatment only (Tables 2 and 3, $p = 0.075$), but an interaction between these two factors was noted ($p < 0.001$). When considering the treatment individually (ASA vs. control), we noted higher GR activity in the infarcted tissue. We noted higher GR activity in the heart tissue collected from the control group of animals, when considering the tissue status individually (infarcted vs. non-infarcted) (Tables 2 and 3).

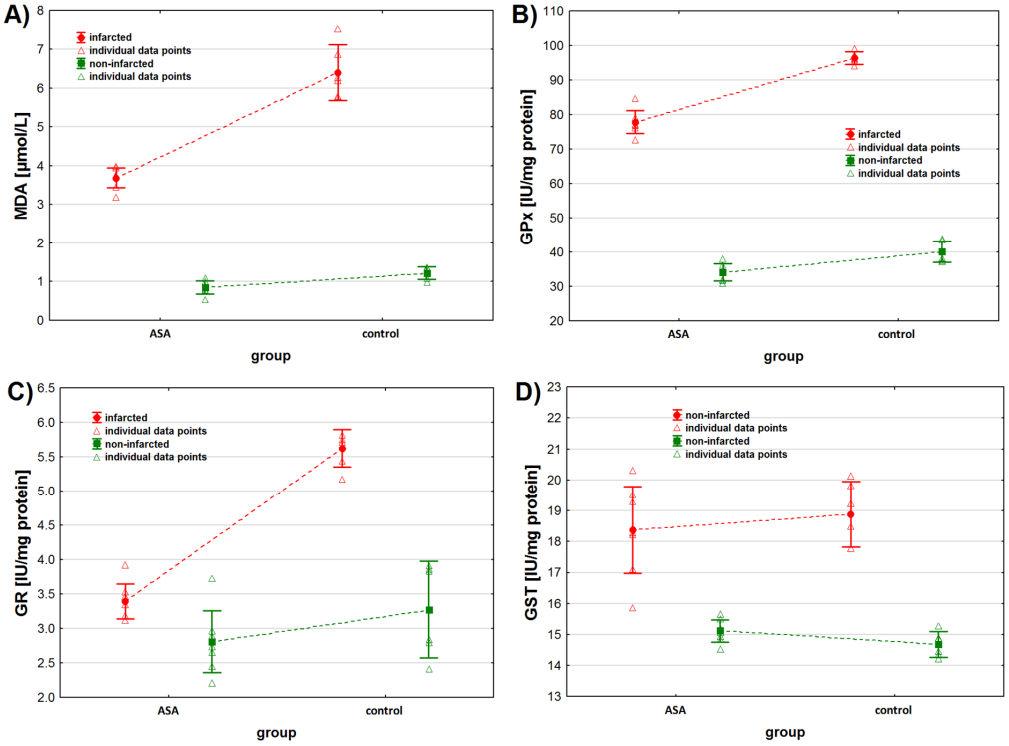

Figure 2. Oxidative stress markers levels in infarcted and the non-infarcted tissues collected from the left ventricle of the heart of pigs (n = 13) subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA): (**A**) malondialdehyde (MDA) concentration [µmol/L], (**B**) glutathione peroxidase (GPx) activity [IU/mg protein], (**C**) glutathione reductase (GR) activity [IU/mg protein] and (**D**) glutathione S-transferase (GST) activity [IU/mg protein]. The results are presented as mean ± 95% confidence interval and triangular markers represent the raw data. For the reader's convenience, the points are connected with dashed lines.

GST activity was higher in the infarcted tissues both in ASA and in the control group (Tables 2 and 3, Figure 2D). We found no differences in GST activity, individually in the infarcted and in the non-infarcted tissue, when comparing the results in the ASA and in the control group.

3.3. Serum

TAC levels depended on the sampling time ($p < 0.001$), when not considering the treatment type, but no differences in TAC levels were found between the ASA and the control group ($p = 0.079$), when considering the treatment type only (Table 4, Figure 3A). However, we found a significant interaction between the treatment type and the treatment time ($p < 0.001$). The lowest TAC levels were observed for both study groups before the infarction, with lower TAC values for the ASA than for the control group except during reperfusion ($p < 0.01$). TAC values increased in both study groups during reperfusion, with the highest values observed for the ASA group ($p < 0.001$). During the recovery period, TAC values decreased in the ASA group, while no change in TAC values was noted in the

control group ($p = 0.073$). Before euthanasia, TAC values in the control group were higher than in the ASA group ($p < 0.001$) (Table 4).

Table 4. Oxidative stress marker levels in the serum of pigs subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA). The results are presented as mean ± standard deviation and were analyzed using two-way ANOVA.

Oxidative Stress Markers	Group	Before Ischemia	During Reperfusion	Before Euthanasia	P_{drug}	P_{time}	$P_{interaction}$
TAC [mmol/L]	ASA ($n = 7$) Control ($n = 6$)	100.9 ± 1.8 107.0 ± 4.5	261.1 ± 19.1 240.9 ± 6.3	221.0 ± 14.9 258.6 ± 20.5	0.079	<0.001	<0.001
TOS [µmol/L]	ASA ($n = 7$) Control ($n = 6$)	41.2 ± 1.8 50.5 ± 2.6	34.8 ± 1.7 40.8 ± 1.0	73.5 ± 3.1 91.1 ± 2.5	<0.01	<0.001	<0.001
OSI [index]	ASA ($n = 7$) Control ($n = 6$)	40.9 ± 2.1 47.3 ± 2.8	13.4 ± 1.0 16.9 ± 0.5	33.4 ± 3.1 35.4 ± 2.7	<0.001	<0.001	0.072
LF [RF]	ASA ($n = 7$) Control ($n = 6$)	19.3 ± 1.9 19.0 ±1.4	10.9 ± 0.8 12.2 ± 0.6	19.1 ± 2.8 12.5 ± 2.0	<0.001	<0.001	<0.001

Abbreviations: ASA—acetylsalicylic acid, LF—lipofuscin, OSI—oxidative stress index, RF—relative lipid extract fluorescence, TAC—total antioxidant capacity, TOS—total oxidative status.

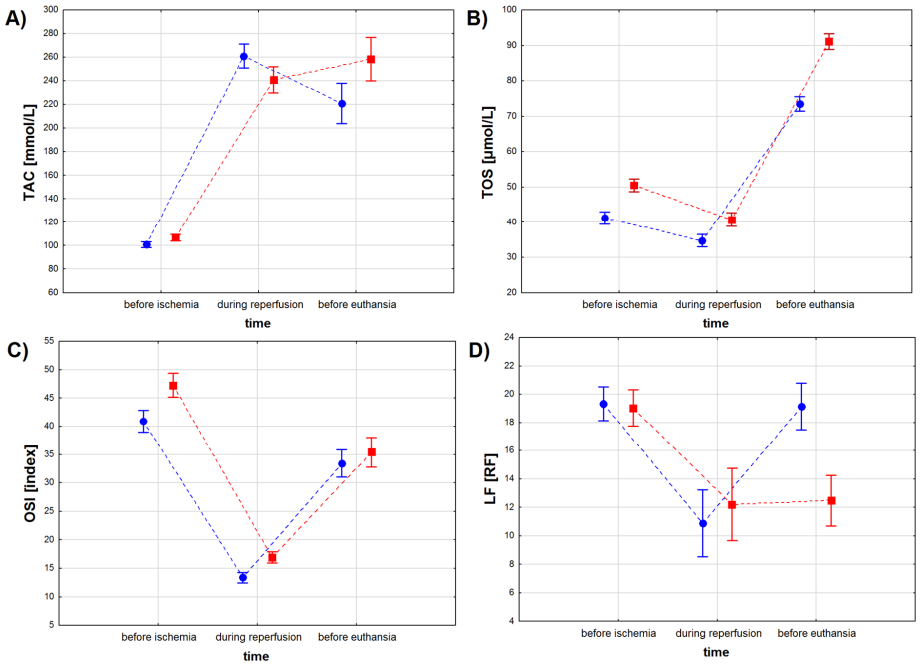

Figure 3. Oxidative stress marker levels in the serum of pigs subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA): (**A**) total antioxidant capacity (TAC) [mmol/L], (**B**) total oxidative status (TOS) [µmol/L], (**C**) oxidative stress index (OSI) [index] and (**D**) lipofuscin (LF) concentration [RF]. The results are presented as mean ± 95% confidence interval and, for the reader's convenience, the points are connected with dashed lines.

TOS values depended on the treatment type and the treatment time, when considering both factors individually and when considering both factors together (Tables 4 and 5, Figure 3B). Higher TOS values were noted for serum sampled from animals from the control group compared to results obtained from the ASA group. Myocardial infarction decreased TOS values in both groups, while the recovery period (sampling before euthanasia) increased TOS values to higher levels than before infarction.

Table 5. Contrast analysis for repeated measures of oxidative stress marker levels in the serum of pigs subjected to a myocardial ischemia-reperfusion protocol assessing the effectiveness of intracoronary administered acetylsalicylic acid (ASA).

Oxidative Stress Markers	$p_{ASA\ vs.\ control}$ before Ischemia	$p_{ASA\ vs.\ control}$ during Reperfusion	$p_{ASA\ vs.\ control}$ before Euthanasia
TAC [mmol/L]	<0.01	<0.001	<0.01
TOS [µmol/L]	<0.001	<0.001	<0.001
OSI [index]	<0.001	<0.001	0.251
LF [RF]	0.766	<0.01	<0.001

Abbreviations: ASA—acetylsalicylic acid, LF—lipofuscin, OSI—oxidative stress index, RF—relative lipid extract fluorescence, TAC—total antioxidant capacity, TOS—total oxidative status.

OSI values depended both on treatment type and treatment time ($p < 0.001$ for both factors), but not on the interaction between these two factors ($p = 0.072$) (Table 4, Figure 3C). OSI values decreased during the reperfusion period and increased during recovery, but to lower values than those noted before ischemia. Before myocardial infarction and during the reperfusion period, OSI values were higher in the control group, while during recovery no statistically significant differences in OSI values were found between the animals injected with ASA and the animals injected with NaCl ($p = 0.251$) (Table 5).

LF concentration depended both on treatment type and treatment time ($p < 0.001$ for both factors) and on the interaction between these two factors (Table 4, Figure 3D). We found no differences in LF concentration between the study groups ($p = 0.766$) before the infarction protocol started (Tables 4 and 5). Higher LF values were noted in the control group ($p < 0.01$) during the reperfusion period and in the ASA group during recovery ($p < 0.001$). We found no differences in LF concentration measured for the reperfusion and for the recovery period in samples collected from the animals from the control group ($p = 0.811$).

4. Discussion

Oxidative stress activates many cellular responses characteristic of heart failure. These changes include not only cellular hypertrophy, changes in gene expression, and cell death [32,33], but also alterations in the turnover and properties of the extracellular matrix [34]. Classic stimuli for ventricular remodeling like wall stress, inflammatory cytokines, and neurohormones (catecholamines and angiotensin II) appear to induce cellular changes partially via oxidative or nitrosative stress [2,35,36]. The pathways activating the various cellular phenotypes of hypertrophy and apoptosis involve one or more stress-responsive protein kinases, many of which are activated by reactive oxygen species (ROS) [33,37]. The present work studied the cardioprotective effect of intracoronary-administered acetylsalicylic acid (ASA) in the ischemia-reperfusion model. The effect was measured with non-enzymatic and enzymatic oxidative stress marker level in the animals' serum and the heart muscle tissue collected from infarcted and non-infarcted parts of the left ventricle. Here, we report that (i) non-enzymatic (TAC, TOS, MDA) and enzymatic (GPx, GR, GST) oxidative stress markers in heart tissue were significantly higher in the control group compared to the ASA group; (ii) intracoronary administered ASA reduced oxidative stress levels compared to the control group injected with NaCl; (iii) ASA showed a protective effect and reduced OS in the infarcted heart tissue when compared to the non-infarcted myocytes of the left ventricle; (iv) TAC, TOS, OSI, GPx, and GR levels were significantly related to the health status of the heart tissue (healthy or infarcted tissue), and type of

drug used (ASA or NaCl in the control group); (v) infarction significantly increased OS in heart tissue compared to healthy heart tissue in the ASA group (except for TOS and GR markers) and in the control group; (vi) despite the type of drug used (ASA or NaCl), infarction decreased or increased TAC, MDA, and GST levels in the heart tissue in the same way. The results obtained for serum showed higher levels of oxidative stress in the control group than in the ASA group and that (vii) time is a significant factor influencing levels of all oxidative stress markers (TAC, TOS, OSI, and LF) measured in animals' serum.

The health status of the animals was evaluated by clinical examination and blood tests at each stage of the experiment. Almost all assayed biochemical and morphological parameters ranged within the reference norms at all experiment stages. Only fibrinogen levels were minimally elevated in control pigs before euthanasia, indicating that the inflammatory process had started. However, we found no differences between the control and ASA groups for health status parameters at any stage of the experiment. Only healthy animals were eligible for the study, and MI induction and ASA treatment did not significantly affect the health status of the pigs.

Despite remarkable improvements in strategies for myocardial infarction and subsequent heart failure treatment, the understanding of heart failure pathogenesis remains limited. The mechanism underlying heart failure in human patients is related to the altered fat tissue amount, inflammatory processes, and changed cardiac physiology that is additionally complicated by comorbidities [38]. Studies by Hill and Singal [39] on the experimental rat model confirmed that cardiac failure and myocardial infarction in rats are related to the deficits in antioxidant capacity and increased oxidative stress.

The potential antioxidant effects of ASA have yet to be fully understood and confirmed. Pratap et al. [40], in a rat model of cerebral ischemia induced by middle cerebral artery occlusion (MCAO), demonstrated that irbesartan (IRB) and ASA combined, used as pretreatment in MCAO rats, elevated the levels of studied antioxidants, glutathione (GSH), superoxide dismutase (SOD) and catalase. In addition, they showed a significant reduction in thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation. The results of their study suggest the antioxidant effects of both IRB and ASA and their synergistic effects. In addition, they described a reduction in brain infarct area volume in animals treated with a combination of IRB and ASA, which may suggest a protective effect of ASA on tissues undergoing ischemia [40]. Berg et al. [41], studying oxidative stress and myocardial injury during coronary artery bypass grafting (CABG), suggested that ASA may exhibit antioxidant effects by enhancing the beneficial effects of the NO axis, inhibiting oxidative stress. This is due to NO's potential as a cyclooxygenase inhibitor, plasma and tissue oxidases inhibitor, and endothelial nitric oxide synthase activator. In this study, levels of 8-iso-PGF2a, which is an end product of arachidonic acid lipid peroxidation and commonly used as a marker of oxidative stress in serum and urine in cardiovascular diseases, were elevated in patients with the preoperative withdrawal of ASA [41]. The potential antioxidant effect of ASA was also suggested by Zhu et al. [42]. The authors demonstrated that both a combination of ASA and clopidogrel and a mixture of ASA with clopidogrel and Xuesaitong (XST) showed antioxidant effects in the course of cerebral ischemia-reperfusion injury. An inhibitory effect was measured with the concentration of the oxidative stress markers 4-HNE (a marker of lipid peroxidation), protein carbonyl (a marker of protein oxidation), and 8-OHdG (a marker of DNA oxidation) [42]. We observed that non-enzymatic (TAC, TOS, MDA) and enzymatic (GPx, GR, GST) oxidative stress markers were significantly higher in the control group than in the ASA group. Intracoronary ASA administration reduced OS, as measured by selected parameters.

The total antioxidant capacity (TAC) assay is designed to measure different elements of the antioxidant defense system and their ability to neutralize oxidative stress [43]. In this study, the TAC level measured in heart muscle was significantly lower in the infarcted tissue than in the non-infarcted tissue and lower in the control group compared to the ASA treated animals. Meanwhile, TAC levels measured in serum depended on the time of blood sampling during the experiment but not on the type of drug used (ASA vs.

NaCl). This shows that ASA had protective effects on the cardiac muscle, significantly improving the different elements of the heart's antioxidant defense system altogether, while results obtained for serum reflect TAC of the whole body. Interestingly, TAC levels in serum were the highest in the ASA group during reperfusion. Spark et al. [44] examined the TAC of patients with chronic critical leg ischemia undergoing femorodistal bypass. They reported that TAC level in the plasma were reduced in chronic critical ischemia, and the patients presented other evidence of free radical damage, as measured by lipid peroxidation and increased vascular permeability. It is known that ASA pretreatment applied before ischemia inhibits the decrease in ATP (adenosine triphosphate) and pH during ischemia [9]. Pretreatment with ASA had a protective effect against myocardial ischemia lasting 30 min, and ASA tended to maintain the myocardial ATP content through ischemia and reperfusion. Moreover, ASA pretreatment also improved acidosis during ischemia. The possible mechanisms of the ASA protective effect are membrane stabilization, inhibition of prostaglandin production, inhibition of lactic acid production, heart rhythm stabilization, and collateral blood flow promotion [11]. However, the molecular basis and cellular mechanisms of ASA's cardioprotective effects remain unknown.

Several reports describing antioxidant protection mechanisms against free radical-induced injury include measuring total antioxidant status (TAS) in body fluids. Surekha et al. [45] reported low TAS levels, compared to controls, in patients with myocardial infarction. Fazendas et al. also showed that low TAS plasma levels in MI patients constituted a risk factor for coronary heart disease [46]. Nojiri et al. also demonstrated significantly lower TAS levels, compared to controls, in 31 patients suffering from coronary artery disease (CAD) [47]. On the other hand, Berg et al. reported elevated TAS levels in two groups of patients, those subjected to percutaneous coronary interventions and coronary angiography [48].

It is challenging to assess antioxidant markers separately due to the various antioxidants present in plasma, serum, urine, or other biological fluids. Thus, TAC and TOS measurements have been used to assess the total status of antioxidant and oxidant systems in organisms [25]. In addition, the oxidative stress index (OSI), the total plasma TOS to TAC ratio, have been used to describe the redox status of oxidation and antioxidation processes [49]. In the present study, OSI and TOS levels were significantly higher in animals from the control group, compared to the ASA group. Similar results were reported by Karabacak et al. [50], who showed that OSI and TAS levels were significantly higher in patients with non-ischemic heart failure compared to control subjects. Also, an elevated OSI level was noted in patients with idiopathic dilated cardiomyopathy [51]. Hill and Singal demonstrated that heart failure subsequent to myocardial infarction was associated with an antioxidant capacity deficit and increased oxidative stress [39]. In addition, CAD patients presented significantly higher OSI and TOS and lower TAC levels than the healthy controls. The authors concluded that oxidative stress is an important element of the early onset CAD pathogenesis, particularly among young smokers [52]. In our study, we observed that TAC was reduced in infarcted heart muscle tissue compared to healthy tissue. However, intracoronary ASA administration significantly increased TAC in heart muscle and serum. We showed that ASA helped to reduce TOS and OSI levels after the infarction-reperfusion event, which confirms its beneficial effect on the oxidative status of heart muscle and serum in the porcine ischemia-reperfusion model.

Lipofuscin is an undegradable material composed of oxidized protein and lipid residues. Its accumulation is proportional to mitochondrial changes as organisms age and the intensity of ROS formation [53]. The present study showed that LF was significantly lower in the ASA group than in the control group, and its lowest level was noted during reperfusion.

Malondialdehyde (MDA) is an important biomarker of oxidative stress, especially lipid peroxidation [54]. It is one of the end products of cell membrane lipid peroxidation, and its content directly reflects the extent of lipid peroxidation and, indirectly, cell damage. Acute myocardial infarction (AMI), accompanied by different levels of ischemic myocardial

injury and necrotic areas, attracts many neutrophils that release ROS. Cardiomyocytes are very ROS-sensitive, as the heart is an energy-consuming organ. Acute myocardial infarction inevitably causes ischemic–anoxic myocardial injury, which induces intensive ROS production [55]. Since MDA levels correlate with AMI severity, this indicator has been used as a determinant of severe coronary diseases [56]. Moreover, it was also shown that lipid peroxidation levels measured in patients' plasma positively correlated with the severity of dilated cardiomyopathy symptoms [57,58]. MDA also reflects coronary artery disease severity and plaque sensitivity [59,60]. Moreover, MDA levels are significantly higher in patients with acute coronary syndromes than in healthy controls and are negatively correlated with these patients' antioxidant status [61]. Finally, Nand et al. [62] reported that MDA levels tripled in AMI patients compared to a control group. Based on MDA levels in the heart tissue, we conclude that intracoronary ASA administration was beneficial for animals subjected to the ischemia-reperfusion procedure.

Myocardium subjected to a ischemia-reperfusion event undergoes complex injury, including injury of the endothelial cells, vascular smooth muscles, conducting tissue, and cardiomyocytes. The endocardium is most prone to these injuries due to local differences in metabolism and energy requirements. Therefore, myocardial injury and tissue necrosis usually originate and expand towards the epicardium. In the myocardium, the oxygen reduction to water happens two ways: primarily via tetravalent reduction of oxygen by the mitochondrial cytochrome oxidase—the pathway generating no intermediates, and in 5% via a univalent reduction that generates ROS such as free radicals: superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($(1)O_2^{\bullet -}$) [63]. In physiological conditions, the antioxidant enzymes are primarily found only in the mitochondria or cytosol. During an ischemic episode, the antioxidant enzymes lose their function and leak into the extracellular fluid, and then the enzymes are washed out during the reperfusion. The washout further depletes the ability to control free radical production. Eventually, the uncontrolled production of free radicals on reperfusion ('respiratory burst') exceeds the activity of antioxidant enzymes, so ROS production is no longer controlled [63]. Changes in GPX, GR, and GST antioxidant enzymes systems may help protect various tissues and cells from oxidative stress. Up-regulation of either of these enzyme systems may protect against oxidative damage during hearts ischemia-reperfusion episodes. We observed an increase in GPX and GR activity in the infarcted tissue compared to non-infarcted heart tissue. That phenomenon may result from the repeated exposure to the mildly elevated ROS levels produced due to augmented demands for ATP under increased workload conditions of the heart. The increased antioxidant production may result in myocardium adaptation and, consequently, mitigate the damage caused by ischemia-reperfusion injury. However, prolonged exposure to oxidative stress was found to deplete the defense systems, including a decrease in GPx activity [64].

The main limitation of the present study is the use only of colorimetric assays to measure oxidative stress effects. By their nature, these assays are subject to confounding by colored biological fluids. Immunohistochemical techniques, such as Western Blotting (e.g., MDA- or 4HNE-positive proteins) or MS-based evaluation (e.g., 8-isoprostanes) could be considered better methods for assessing the effects of oxidative stress in tissues. However, the more cost-effective and less time-consuming colorimetric methods allow a lot of data to be gathered from a few animal samples.

5. Conclusions

The present study demonstrated that increased in vivo lipid peroxidation, measured with malondialdehyde (MDA) and lipofuscin (LF), decreased antioxidants, total antioxidant capacity (TAC) and total oxidative status (TOS), and increased enzyme activity, glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST) in infarcted heart tissue and serum are related to myocardial ischemia-reperfusion injury. Intracoronary-administered acetylsalicylic acid (ASA) alleviated the oxidative stress increase, expressed by increased oxidative stress markers and decreased oxidative stress

index (OSI). The cardioprotective effect of intracoronary-administered ASA proved in the porcine model gives foundations for developing new therapies for treating ischemia-reperfusion complications in humans.

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Institutional Review Board Statement: The study was conducted according to the National Institute of Health guidelines for the care and use of laboratory animals and approved by the Local Ethics Committee for Animal Experiments at Wroclaw University of Environmental and Life Sciences, Poland (protocol no. 081/2019, approved on 11 December 2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data it is not publicly available because supporting data cannot be made available openly due to the owning property nature.

Conflicts of Interest: The authors declare no conflict of interest.

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WNIOSKI

1. Uszkodzenie niedokrwienno-reperfuzyjne mięśnia sercowego skutkuje nasileniem stresu oksydacyjnego wyrażonym przez zwiększenie peroksydacji lipidów, osłabienie nieenzymatycznej obrony antyoksydacyjnej organizmu i zwiększenie aktywności enzymów o działaniu antyoksydacyjnym.

2. Dowieńcowe podanie kwasu acetylosalicylowego wykazało działanie kardioprotekcyjne i zmniejszyło poziom stresu oksydacyjnego w tkance mięśnia sercowego świń otrzymujących kwas acetylosalicylowy w porównaniu z grupą kontrolną, co jest wyrażone zwiększeniem całkowitej zdolności antyoksydacyjnej, zmniejszeniem całkowitego statusu oksydacyjnego i wskaźnika stresu oksydacyjnego, niższym stężeniem dialdehydu malonowego oraz niższą aktywnością peroksydazy glutationowej, reduktazy glutationowej i S-transferazy glutationowej w tkankach pobranych z mięśnia sercowego świń, którym dowieńcowo podano kwas acetylosalicylowy.

3. Całkowita zdolność antyoksydacyjna w tkankach pobranych z mięśnia sercowego świń poddanych procedurze wywołania niedokrwienia i zawału mięśnia sercowego jest wyższa w grupie świń otrzymujących kwas acetylosalicylowy w porównaniu z grupą kontrolną, co oznacza, że kwas acetylosalicylowy podany dowieńcowo może być rozważany jako lek modyfikujący przebieg uszkodzenia niedokrwienno-reperfuzyjnego mięśnia sercowego poprzez korzystny wpływ na status oksydacyjny mięśnia sercowego.

4. Dowieńcowe podanie kwasu acetylosalicylowego wykazało działanie kardioprotekcyjne i zmniejszyło poziom stresu oksydacyjnego w surowicy zwierząt otrzymujących kwas acetylosalicylowy w porównaniu z grupą kontrolną, co jest wyrażone zwiększeniem całkowitej zdolności antyoksydacyjnej, zmniejszeniem całkowitego statusu oksydacyjnego i wskaźnika stresu oksydacyjnego oraz niższym stężeniem lipofuscyny w surowicy świń, którym dowieńcowo podano kwas acetylosalicylowy.

5. Okluzja proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej, prowadząca do niedokrwienia mięśnia sercowego, charakteryzuje się wysoką wywoływalnością komorowych zaburzeń rytmu serca u świń poddanych procedurze wywołania niedokrwienia mięśnia sercowego, a znaczny odsetek arytmii komorowych

wywołanych poprzez okluzję proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej stanowią częstoskurcz komorowy i migotanie komór, będące bezpośrednim zagrożeniem życia.

6. Zmodyfikowane postępowanie anestetyczne, w tym stabilizacja zaburzeń hemodynamicznych rozwijających się na skutek niedokrwienia mięśnia sercowego i towarzyszących niedokrwieniu mięśnia sercowego arytmii, wpływa na 100 % przeżywalność świń poddanych procedurze wywołania niedokrwienia i zawału mięśnia sercowego poprzez okluzję proksymalnego odcinka gałęzi przedniej zstępującej lewej tętnicy wieńcowej.

PODSUMOWANIE

W cyklu artykułów, które składają się na pracę doktorską, przedstawiono badania mające na celu ocenę kardioprotekcyjnego wpływu kwasu acetylosalicylowego na mięsień sercowy poddany uszkodzeniu niedokrwienno-reperfuzyjnemu oraz analizę arytmii rozwijających się w trakcie MI u świń poddanych procedurze niedokrwienia mięśnia sercowego w zmodyfikowanym protokole anestetycznym. Kardioprotekcyjne działanie ASA oceniano poprzez analizę statusu oksydacyjnego i pomiar wybranych markerów stresu oksydacyjnego w tkankach pobranych z mięśnia sercowego oraz w surowicy świń poddanych procedurze MI. Dzięki przeprowadzonym doświadczeniom uzyskano stabilny świński model niedokrwienia i zawału mięśnia sercowego i uszkodzeń niedokrwienno-reperfuzyjnych, który cechuje się wysokim odsetkiem generowanych arytmii komorowych i 100% przeżywalnością. Wykazano także, że kwas acetylosalicylowy podawany dowieńcowo w trakcie niedokrwienia mięśnia sercowego, ma działanie kardioprotekcyjne związane ze zmniejszeniem wzrostu stresu oksydacyjnego w surowicy i tkankach pobranych z serc badanych świń.

Uzyskane wyniki mogą stanowić podstawę dalszych badań dotyczących dowieńcowego podawania ASA, zwłaszcza podczas epizodów ostrego niedokrwienia mięśnia sercowego, co może przyczynić się do opracowania nowych terapii mających zastosowanie w leczeniu powikłań niedokrwienno-reperfuzyjnych u ludzi. Opracowany świński model MI, ze względu na wysoką przeżywalność zwierząt, może posłużyć jako model do dalszych badań nad komorowymi zaburzeniami rytmu serca w przebiegu MI i uszkodzenia niedokrwienno-reperfuzyjnego mięśnia sercowego u ludzi.