Abstract. Tetrandrine, the active principle of Stephania tetrandra radix extracts, has broad pharmacological activity, including effects on the cardiovascular system: it has been shown to reduce the size of acute myocardial infarction in rats undergoing coronary vessel ligation and to improve heart lesions in the constriction/reperfusion model by means of mechanisms involving peroxidation, calcium antagonism and coagulation. The aim of this study was to investigate whether tetrandrine has anti-infarction, antioxidant and anticoagulant effects in rabbits treated with isoproterenol, a drug capable of causing peroxide generation, calcium overload and coagulation alterations, and inducing myocardial infarction. The results showed that pretreatment with tetrandrine protects against the myocardial injuries caused by isoproterenol. It counteracted the appearance of myocardial necrotic lesions and ischemic electrocardiographic modifications, such as ST segment alterations, prevented the appearance of the plasma cardiac necrosis markers c-troponin I and myoglobin, lowered malondialdehyde levels, and prolonged partial thromboplastin time. The protective effects of tetrandrine can be attributed to its antioxidant action in lowering peroxide levels and its ability to counteract coagulating activity. Tetrandrine seems to offer full protection against myocardial infarction experimentally induced by the non-invasive treatment of rabbits with isoproterenol.

Tetrandrine is a pharmacologically active alkaloid isolated from the plant Stephania tetrandra (1), which is used in traditional Chinese medicine as an antipyretic or analgesic compound (1), and is now extensively studied as a tumour cell cytotoxic agent and angiogenesis inhibitor (2). Some studies have also shown that it has antihypertensive activity (3-6) and antiarrhythmic effects (7). Its cardiovascular activity seems to be particularly promising and deserves great attention.

It has been more recently shown that extracts of S. tetrandra and its active principle tetrandrine have anti-infarction activity: they decrease the incidence of arrhythmias and infarct size in isolated rat hearts following coronary vessel obstruction (8), have protective effects in rats subjected to acute coronary occlusion and reduced the infarcted area (9), reduce the area of myocardial infarction in rats undergoing myocardial ischemia and reperfusion (10), and myocardial reperfusion injuries in rats undergoing coronary ligation followed by reperfusion (11). The above studies indicate that various mechanisms underlie the cardiac anti-ischemic effects of tetrandrine. According to Shen et al., tetrandrine improves ischemia/reperfusion injury in rats by inhibiting neutrophil priming and activation, and thus abolishing the subsequent infiltration and reactive oxygen species production that causes myocardial reperfusion injury (11); according to Yu et al. (10), the extract containing tetrandrine reduces infarct size by acting through a mechanism involving channel antagonism (10). Other authors have shown that tetrandrine also has anti-thrombotic activity by inhibiting platelet aggregation (12). In other words, tetrandrine may counteract the damage induced by coronary ischemia by means of antioxidant (11) or calcium antagonist (10) or anticoagulation mechanisms (12).

The aim of this study was to investigate whether tetrandrine has anti-infarction, antioxidant and anticoagulant effects in rabbits treated with isoproterenol, a drug capable of causing peroxide generation (13, 14), calcium overload (15) and coagulation alterations (14, 15), and inducing myocardial infarction (14, 15).

To this end, we evaluated ECG alterations, histological lesions and the appearance of plasma cardiac necrosis markers, and verified whether the administration of tetrandrine could...
counteract the altered plasma malondialdehyde (MDA) levels and partial thromboplastin time (PTT) observed in isoproterenol-treated animals.

**Materials and Methods**

**Materials, reagents and animal treatment.** Isoproterenol and tetrandrine were purchased from Sigma (Milan, Italy); the reagents and instruments for evaluating c-troponin I and myoglobin came from Dade-Behring (Milan, Italy), and the reagents for measuring PTT came from Boehringer-Roche (Milan, Italy).

The study was carried out in conformity with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twenty-four male New Zealand white rabbits weighing 3000±100 g were obtained from Harlan (Correzzana, Milan, Italy), and divided into groups of six animals each. One group was treated with isoproterenol 3 mg/kg i.p., the second with tetrandrine 15 mg/kg i.m., and the third received a combination of tetrandrine 15 mg/kg i.m. and isoproterenol 3 mg/kg i.p., with the tetrandrine being given ten minutes before the administration of isoproterenol. The fourth group received saline and acted as a control group.

Before treatment, the rabbits were anesthetized with urethane (700 mg/kg i.p.), with clonazepam (0.125 mg/kg i.p.) being given 30 minutes later. They were sacrificed 60 minutes after isoproterenol administration.

**Electrocardiography.** The electrocardiograms were recorded using an Electrocardiograph 3C (Elettronica Trentina, Trento, Italy) and ten leads: I, II, III, aVR, aVL, aVF and precordial VM (instead of V1, V2, V3), V4, V5 and V6. The recordings were made at baseline (with the animals anesthetized before starting the experiments), and then for one minute every five minutes during treatment, and the types of alterations (ST segment elevation or depression, and T wave inversion) were evaluated. In particular, the alterations observed in the last trace (recorded five minutes before sacrifice) were carefully analyzed because they would be closest to any altered plasma biochemical profiles immediately after collection. Immediately before sacrifice, blood was collected from all of the animals using lithium-heparin (14 IU/ml) or citrate and the plasma was prepared and stored at −20°C until assay.

**Plasma cardiac markers.** As it has been shown that polyclonal antibodies specifically prepared against human cardiac c-troponin I react with c-troponin I in the supernatants of heart homogenates of rabbits and other species (16), c-troponin I was evaluated in rabbit lithium-heparin plasma using a commercially available Dade-Behring reagent for human c-troponin I (17, 18), in which monoclonal antibodies are conjugated with alkaline phosphatase acting on the fluorogenic 4-methyl-umbelliferil-phosphate substrate. Myoglobin was evaluated in plasma using a Dade-Behring reagent in which the anti-myglobin monoclonal antibody interacts with the myoglobin of the plasma sample to give a turbidimetric reaction (19).

**Histology.** Immediately after the sacrifice of the anesthetized animals, their hearts were removed and immediately fixed in 10% buffered formalin before being processed for histological examination. The ventricular mass was sectioned from the apex to the base of the heart in order to obtain 0.4 cm thick transverse slices, which were then embedded in paraffin after being dehydrated in alcohol (from 80% to absolute alcohol) and subsequently cleared with xylene. Five-micron thick serial histological sections were obtained from the paraffin blocks and stained with hematoxylin and eosin as previously described (20).

The histological findings are referred to in order to describe the sites of the lesions; no morphometric evaluations were made in this study. The photomicrographs were shot using a Zeiss MC 80 Axioscope photomicroscope at different magnifications (×100, ×200 and ×400).

**Free plasma MDA.** Free plasma MDA was extracted without hydrolysis, and assayed using the gas chromatography-mass spectrometry method described by Cighetti et al. (21).

**Pro-coagulant activity.** PTT was assayed in citrate plasma using a standard procedure (22). The plasma was diluted 1:10 with 0.05 M phosphate buffer at pH 7.4.

**Statistical analysis.** The values of c-troponin I, myoglobin, MDA and PTT were analyzed using analysis of variance, and their statistical significance was evaluated using Tukey’s test (23). The control and isoproterenol groups were compared with each other, and with the tetrandrine or tetrandrine plus isoproterenol groups. Statistical significance was assumed at p<0.05.

**Results**

**General observations.** Treatment with isoproterenol, and pretreatment with tetrandrine have different effects on myocardial histology, electrocardiographic profiles, plasma cardiac necrosis markers, free plasma MDA and coagulation parameters.

**Electrocardiographic data.** Isoproterenol administration caused ischemic lesions evaluated as ST segment alterations in DIII, aVF, VM, V4, V5 and V6. Pretreatment with tetrandrine affected the electrocardiographic profiles of the isoproterenol-treated animals insofar as no ST alterations or T wave inversions were observed in the animals receiving combined tetrandrine/isoproterenol treatment (Figure 1).

**Plasma cardiac necrosis markers.** The mean plasma c-troponin I level was 0.03±0.01 ng/ml in the controls, 5.98±1.29 ng/ml in the isoproterenol group, 0.11±0.04 ng/ml in the tetrandrine group, and 1.8±0.76 ng/ml in the animals receiving tetrandrine plus isoproterenol. The levels in the isoproterenol group were significantly higher (p<0.001) than those observed in all of the other groups, and treatment with tetrandrine very significantly lowered c-troponin I levels in the tetrandrine plus isoproterenol group (p<0.001).

The mean plasma myoglobin level was 2.6±0.25 ng/ml in the controls, 189.5±26.5 ng/ml in the isoproterenol group, 4.3±0.8 in the tetrandrine group, and 18.75±4.23 in the group receiving tetrandrine plus isoproterenol. The levels in the isoproterenol group were significantly higher (p<0.001) than those observed in all of the other groups, and treatment with tetrandrine very significantly lowered myoglobin levels in the tetrandrine plus isoproterenol group (p<0.001).
Histological findings. The histological sections obtained from the hearts of the animals receiving isoproterenol alone showed various degrees of interstitial hemorrhage, presence of necrotic areas with dead myocells and vacuolar phenomena (I₁ and I₂), wavy fibres (I₃) areas with contraction band necrosis (I₄) (Figure 2).

None of the histological images obtained from the animals pretreated with tetrandrine showed any remarkable alterations (T+I) (Figure 2). The histological profile of animals receiving tetrandrine alone appear similar to control (Figure not shown).

Free plasma malondialdehyde. The mean free plasma malondialdehyde level was 1.93±0.22 μmol/l in the controls, 4.15±0.38 μmol/l in the isoproterenol group, 1.97±0.21 μmol/l in the tetrandrine group, and 2.71±0.32 μmol/l in tetrandrine plus isoproterenol group. The levels in the isoproterenol group

Figure 1. Electrocardiograms of control, isoproterenol-, tetrandrine and isoproterenol-treated rabbits recorded using 3-channel electrocardiograph, with a calibration of 10 mm/mV and a chart speed of 50 mm s⁻¹. I: Isoproterenol-treated animals: ischemic lesions appear (ST elevation in DIII; aVF, VM, V₄, V₅, V₆); T+I: tetrandrine and isoproterenol administration: the tracings appear similar to those of the control animals. In animals treated with tetrandrine alone the tracings appear similar to those of the control and have been omitted. The heart rate of 330 bpm shown here observed in the isoproterenol-treated rabbit was higher than that observed in all other tracings.
were significantly higher than those observed in all of the other groups \((p<0.001)\), and treatment with tetrandrine significantly lowered MDA levels in the tetrandrine plus isoproterenol group \((p<0.05)\).

**PTT values.** The PTT values were 38.3\(\pm\)2.12 for controls, 30.1\(\pm\)1.84 with isoproterenol, 49.8\(\pm\)2.31 with tetrandrine and 51.5\(\pm\)5.12 seconds with tetrandrine plus isoproterenol.

The PTT values found in isoproterenol treated rabbits appeared to be shorter than in controls \((p<0.05)\), tetrandrine-treated \((p<0.001)\) and tetrandrine plus isoproterenol-treated \((p<0.001)\) rabbits. The treatment with tetrandrine increased the PTT values in tetrandrine-treated and in tetrandrine plus isoproterenol-treated rabbits vs. controls \((p<0.01)\) and vs. rabbits treated with isoproterenol alone \((p<0.001)\).

**Discussion**

The administration of isoproterenol and tetrandrine, alone and in combination, has different effects on the parameters related to myocardial infarction: ECG findings, plasma c-troponin I and myoglobin levels, myocardial histology, and biochemical indicators such as plasma MDA levels and PTT. Isoproterenol treatment affected the electrocardiographic profile. The tracings of the animals receiving isoproterenol alone showed both T-wave inversions and ST-segment alterations throughout the duration of the experiment, thus suggesting the presence of ischemic myocardial damage (Figure 1). These ECG alterations are supported by the observation that the administration of isoproterenol caused the appearance of plasma c-troponin I and myoglobin, which are markers of cardiac necrosis.

The increased levels of heart injury markers after isoproterenol treatment are in line with reports by us and others concerning rats treated with isoproterenol or orciprenaline (14, 15, 24-26). Furthermore, these markers have previously been detected in rabbit plasma, which can be carried out using antibodies against human c-TnI because the peptide sequences in human and rabbit cardiac troponins are highly similar (16-18), and consequently give cross-reactivity reactions (16, 18).

The appearance of plasma cardiac necrosis markers was accompanied by histological alterations. As can be seen in Figure 2, heart histology revealed vacuoles within the cytoplasm, and the presence of edema and aspects of hydropic change and degeneration. Hydropic degeneration is due to decreased membrane ionic pump activity as a result of the lack of ATP synthesis, which also causes intracellular water accumulation and can be attributed to peroxidation-induced cell structure damage (27).

The histological examination also showed areas of coagulative necrosis in the isoproterenol-treated animals that are characteristic of the peroxide-induced death of myocardial cells (27).

Wavy fibres and contraction band necrosis are found at the periphery of an infarcted area and are attributable to systolic tugs of viable fibres adjacent to non-contractible fibres (27). We found contraction bands in our isoproterenol-treated animals and these were probably due to the peroxidation stress induced by catecholamines (28, 29). The heart alterations were
accompanied by high levels of peroxide generation, as shown by the increase in peroxide values measured by means of the MDA peroxidation marker (30). The catecholaminergic activation induced by isoproterenol favours the formation of hydroxyl radicals (31).

Oxidative damage begins when the double bonds of unsaturated fatty acids of lipids membrane are attacked by oxygen-derived free radicals, particularly OH-, thus giving rise to MDA and other oxidation products (32). Unstable and reactive peroxides can cause extensive membrane organelle and cell damage, with loss of membrane integrity, as well as mitochondrial lesions (33), and mitochondrial dysfunction leads to ATP and energy substrate depletion and massive contraction band necrosis (34).

It has been reported that isoproterenol causes calcium channel opening (35), calcium overload, the loss of energy substrates, and myocardial necrosis (36, 37); peroxide generation seems to act synergistically with calcium overload to induce myocardial necrosis (37) in isoproterenol-treated animals.

In our experiments, tetrane had a protective effect against infarction and its associated signs. It protected against electrocardiographic changes as there were no signs of the ST segment or T wave alterations indicative of ischemia (Figure 1); it prevented the appearance of myocardial lesions as the histological findings were normal, and there was no observable necrosis (Figure 2); it blocked the appearance of plasma cardiac necrosis markers c-troponin I and myoglobin because, as the myocardium was not damaged even in the presence of isoproterenol, no cardiac necrosis markers were released into the plasma.

It is important to note that tetrane lowered the plasma MDA levels in animals receiving isoproterenol.

Tetrane may have protected the animals because of its antioxidant action (38). It scavenges superoxide anions (38) and blocks the conditions leading to the generation of the hydroxyl radical .OH (39). Furthermore it improves ischemia/reperfusion lesions in rat myocardium by inhibiting the neutrophil activation and infiltration and ROS production that cause myocardial reperfusion injury (11). Tetrane may also have protected the animals by interacting with slow calcium channels (40). Finally, isoproterenol shortens PTT as a result of adrenoreceptor stimulation (41) and peroxide production (42), and the administration of tetrane alone or in combination with isoproterenol significantly prolonged PTT. By means of its peroxide-scavenging activity (38), tetrane counteracts the increase in the levels of the peroxidation marker MDA, and this may prolong the shortened PTT caused by isoproterenol. As the conversion of prothrombin to thrombin is accelerated by the addition of calcium ions that interact with the carboxyl groups of glutamic acid of factor X and factor II (prothrombin) (43), tetrane may interfere with the calcium-binding sites on the coagulation factors, as it may interact with the calcium and glutamic acid residues located in calcium channels (40, 44). It therefore contributes to protection against myocardial infarction by removing the procoagulant activity induced by isoproterenol.

In conclusion, we found that tetrane seems to prevent experimental myocardial infarction induced in intact animals by a non-invasive technique (14, 15), and seemed to protect against myocardial necrosis by means of mechanisms possibly involving the removal of peroxides and the inhibition of coagulant activity. It may therefore be suitable for development as a protective drug in patients affected by ischemic coronary diseases associated with sympathetic oxidative stress or thrombotic events.

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References


