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OXYGEN RADICAL INJURY AND LOSS OF HIGH-ENERGY COMPOUNDS IN ANOXIC AND REPERFUSED RAT HEART: PREVENTION BY EXOGENOUS FRUCTOSE-1,6-BISPHOSPHATE

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Isolated Langendorff-perfused rat hearts after 10 minutes preperfusion, were subjected to a substrate-free anoxic perfusion (20 minutes) followed by 20 minutes reperfusion with a glucose-containing oxygen-balanced medium. Under the same perfusion conditions, the effect of exogenous 5 mM fructose-1,6-bisphosphate has been investigated. The xanthine dehydrogenase to xanthine oxidase ratio, concentrations of high-energy phosphates and of TBA-reactive material (TBARS) were determined at the end of each perfusion period in both control and fructose-1,6-bisphosphate-treated hearts. Results indicate that anoxia induces the irreversible transformation of xanthine dehydrogenase into oxidase as a consequence of the sharp decrease of the myocardial energy metabolism. This finding is supported by the protective effect exerted by exogenous fructose-1,6-bisphosphate which is able to maintain the correct xanthine dehydrogenase/oxidase ratio by preventing the depletion of phosphorylated compounds during anoxia. Moreover, in control hearts, the release of lactate dehydrogenase during reperfusion, is paralleled by a 50% increase in the concentration of tissue TBARS. On the contrary, in fructose-1,6-bisphosphate-treated hearts this concentration does not significantly change after reoxygenation, while a slight but significant increase of lactate dehydrogenase activity in the perfusates is observed.

On the whole these data indicate a direct contribution of oxygen-derived free radicals to the worsening of post-anoxic hearts. A hypothesis on the mechanism of action of fructose-1,6-bisphosphate in anoxic and reperfused rat heart and its possible application in the clinical therapy of myocardial infarction are presented.

KEY WORDS: Anoxia, reperfusion, rat heart, fructose-1,6-bisphosphate

INTRODUCTION

Tissue injuries to anoxic and/or ischaemic heart may be mainly related to an imbalance between production and consumption of energy. This is due to the progressive decrease of mitochondrial oxidative metabolism, on which the maintainance of physiological concentrations of high-energy phosphorylated compounds is mainly

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based.[1,2] Among the numerous alterations induced by anoxia and ischaemia, three phenomena which reportedly affect the successive phase of normoxic reperfusion are: i) the accumulation of the end products of ATP catabolism, hypoxanthine and xanthine;[3] ii) the increase of cytosolic free Ca^{2+} which leads to the activation of Ca^{2+}-dependent proteases;[4] iii) the irreversible transformation of xanthine dehydrogenase (XDH) into xanthine oxidase (XO) catalyzed by the Ca^{2+}-activated proteases.[5-7]

Due to this sequence of events reperfusion is generally characterized by an increase of tissue damage. This damage appears to be related, at least in part, to oxygen-derived free-radicals which may be produced by anoxic or ischaemic tissues[6-8] during exposure to oxygen. Thus, in the conversion of xanthine into uric acid, catalyzed by XO, molecular oxygen may undergo a single electron reduction[9] that generates a flow of superoxide anions which may be further transformed into more dangerous free-radical species.[10]

Although data concerning the irreversible transformation of XDH into XO and oxygen radical-related damage (such as lipid peroxidation) in the post anoxic and/or post-ischaemic heart have been reported by several authors,[5-7,11] the involvement of oxygen-derived free-radicals in the ontogenesis of reperfusion injuries is still under debate. However, it has been established that reperfusion, although unavoidable for the salvage of the heart, is often characterized by an incomplete recovery of myocardial functions and metabolism, both parameters being strongly influenced by the duration of anoxia or ischaemia.[12,13] Furthermore, it is clear that the maintainance of adequate levels of high-energy metabolites during anoxia is crucial both for the recovery of mechanical functions and for the prevention of the onset of the so called oxygen paradox. In this context, it is worthwhile to mention that exogenous fructose-1,6-bisphosphate (F-1,6-P_2) has been reported to reduce superoxide production from human polymorphonuclear leucocytes stimulated by phorbol esters,[14] to ameliorate some haemodynamic and electrocardiographic parameters of patients suffering from acute myocardial infarction[15] and to prevent cardiotoxicity induced by subchronic doxorubicin administration in mice.[16]

The aim of our study was to verify the irreversible transformation of XDH into XO and to evaluate the oxygen radical-induced lipid peroxidation (measured as TBA-reactive substances) in isolated Langendorff-perfused rat hearts subjected to anoxia and/or reperfusion. In addition, we determined whether exogenous F-1,6-P_2, by preventing the depletion of high-energy phosphate compounds during anoxia, might inhibit the onset of the oxygen radical-related reperfusion injury.

MATERIALS AND METHODS

Male Wistar rats of 300–350 g/b.w. were used. Animals were anaesthetized by i.p. injection of thiopentale 150 mg/kg b.w.. Hearts were quickly removed and the aorta was cannulated. Spontaneously beating hearts were perfused by the non-recirculating Langendorff technique[17] with a Krebs-Ringer solution containing 11 mM glucose, 2.5 mM $CaCl_2$ and 12 U/l of insulin at a constant hydrostatic pressure of 7.85 Kpa (80 cm H_2O). The perfusion medium was equilibrated at 37°C with 95% O_2 + 5% CO_2. After 10 minutes reperfusion (step 1), hearts were subjected to 20 minutes of anoxic perfusion with a glucose-free nitrogen balanced (95% N_2 + 5% CO_2) Krebs-Ringer (step 2), followed by 20 minutes of normoxic reperfusion with the starting

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buffer (step 3). F-1,6-P_2-treated hearts were perfused under the same experimental conditions except that 5 mM F-1,6-P_2 (Esafosfina®, Biomedica Foscama, Rome, Italy) pH 7.3 was always present in the perfusion media throughout the different perfusion steps (preperfusion, anoxic perfusion, normoxic reperfusion). At the end of each step, both control and F-1,6-P_2-treated hearts were freeze-clamped, cooled in liquid nitrogen and processed either for the determination of enzymatic activities[16] and TBA-reactive material[16] or for the analyses of metabolites.[18] Xanthine dehydrogenase and xanthine oxidase were determined according to.[19] TBA-reactive material was assayed by the thiobarbituric acid reaction[20] using 1,1,3,3-tetraethoxypropane as an external standard (Sigma, St. Louis, Mo., U.S.A.). ATP,[21] ADP,[21] creatine phosphate (CrP)[22] and creatine (Cr)[22] were determined enzymatically whilst inorganic phosphate (Pi) was assayed colorimetrically.[23] Lactate dehydrogenase activity (LDH)[21] was determined in separately collected perfusates relative to the three perfusion steps. Enzymes, coenzymes and substrates for metabolite analyses and for enzyme assay were purchased from Boehringer (Mannheim, FRG); all other reagents were of the highest purity available from commercial sources. All the spectrophotometric assays were carried out by a Jasco 650 double beam spectrophotometer (Tokyo, Japan). Within group and between group comparisons were performed by a one-way analysis of variance.

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RESULTS

The parameters reflecting the energy state of myocardial tissue both of control and F-1,6-P_2-treated hearts are reported in Table I. No differences were observed for any parameter after the normoxic preperfusion period. Comparison of the values obtained at the end of step 2 indicates that F-1,6-P_2 administration to anoxic hearts significantly decreases the depletion of high-energy substrates. In particular, the concentrations of ATP and CrP in F-1,6-P_2-treated hearts were 2.4 and 1.9 fold higher respectively than the corresponding values of control hearts ($p < 0.01$ and $p < 0.05$ respectively), while the Cr values were significantly higher ($p < 0.05$) in the control group. As a consequence, the phosphorylation potential of F-1,6-P_2-treated hearts, calculated from the equilibrium constant of the creatine kinase reaction according to Nuutinen et al.,[24] was 3.6 fold higher than in the control hearts recorded at the same step ($p < 0.001$). This parameter was calculated by assuming that no change in intracellular pH occurred during anoxic perfusion.[25]

The maximal effectiveness of the drug towards high-energy metabolites was obtained after 20 minutes of normoxic reperfusion. In fact, F-1,6-P_2 treatment restored to physiological levels the concentration of CrP ($p < 0.001$ compared to the same step of control hearts) and induced a marked increase in the ATP concentration which, although different from the value recorded at the end of preperfusion ($p < 0.05$), was 2.25 fold higher than that of control hearts at the end of reperfusion ($p < 0.001$).

For control hearts the values of the XDH/XO ratio (see Figure 1) at the end of anoxia and of reperfusion were 5.7 and 12 fold lower, respectively, than the corresponding value recorded after 10 minutes preperfusion ($p < 0.001$). Conversely, the XDH/XO ratio in the F-1,6-P_2-treated hearts indicated that the irreversible transformation of XDH into XO did not occur after step 2 or step 3 (Figure 1). Presumably,

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TABLE I

Protective effect on phosphorylated compounds of anoxic and reperfused rat heart by 5 mM fructose-1,6-bisphosphate

	Step	ATP	ADP	CrP	Cr	P_i	Phosphorylation potential $[ATP]_f/[ADP]_f \times [P_i]$
CONTROL HEARTS	1	23.19 (1.97)	7.97 (0.55)	32.08 (1.52)	23.00 (2.00)	16.12 (2.53)	72.57 (10.33)
	2	4.76 (1.52)	7.48 (1.35)	6.32[c] (2.00)	37.81[c] (4.02)	23.80[c] (3.00)	5.05[c] (0.72)
	3	9.03[cd] (0.98)	7.01 (1.22)	12.85[ce] (2.35)	28.81[bg] (3.11)	20.02 (5.81)	23.14[cf] (3.40)
$F-1,6-P_2$-TREATED HEARTS	1	23.61 (2.03)	7.73 (1.70)	32.81 (3.03)	22.85 (2.11)	18.26 (3.18)	69.86 (9.27)
	2	11.54[ch] (1.78)	7.12 (1.84)	12.14[cg] (2.45)	29.61[bg] (2.84)	20.45 (3.70)	18.36[ci] (2.00)
	3	20.32[afi] (2.28)	7.23 (1.08)	28.79[fi] (5.17)	22.91[eg] (2.55)	21.00 (3.39)	57.07[fi] (11.88)

Values are the mean (s.d.) of six different hearts per each step both for control and $F-1,6-P_2$-treated hearts.

The parameters are expressed as μmoles/g d.w., out of the phosphorylation potential which is calculated as in (24) and expressed as $10^3 \times M^{-1}$.

Step 1 = 10 minutes preperfusion; Step 2 = 20 minutes anoxic perfusion, and Step 3 = 20 minutes normoxic reperfusion. Perfusion conditions are fully explained under Materials and Methods.

a) significantly different from Step 1 of the same group of hearts ($p < 0.05$)
b) significantly different from Step 1 of the same group of hearts ($p < 0.01$)
c) significantly different from Step 1 of the same group of hearts ($p < 0.001$)
d) significantly different from Step 2 of the same group of hearts ($p < 0.05$)
e) significantly different from Step 2 of the same group of hearts ($p < 0.01$)
f) significantly different from Step 2 of the same group of hearts ($p < 0.001$)
g) significantly different from corresponding Step of control hearts ($p < 0.05$)
h) significantly different from corresponding Step of control hearts ($p < 0.01$)
i) significantly different from corresponding Step of control hearts ($p < 0.001$)

this was related to the maintainance of a high energy state in the hearts supplemented with 5 mM $F-1,6-P_2$.

The direct irreversible change of XDH into XO observed in control hearts is probably directly responsible for the 51.4% increase of TBA-reactive material (TBARS) after normoxic reperfusion ($p < 0.001$) with respect to the preperfusion value (Figure 2). On the contrary, $F-1,6-P_2$-treated hearts maintained a concentration of TBARS similar to that recorded at the end of preperfusion ($p < 0.001$ with respect to step 3 of control hearts).

Figure 3 reports the lactate dehydrogenase activity determined in the perfusates after the three steps in both control and $F-1,6-P_2$-treated hearts. The maximum increase was recorded in the perfusates of control hearts at the end of reperfusion (7 fold in comparison with the preperfusion value, $p < 0.001$). In parallel, $F-1,6-P_2$-treated hearts displayed a similar trend although much less marked (see Figure 3).

In Figure 4 the inhibition of the heart rate as a function of the perfusion time in both control and in $F-1,6-P_2$-treated hearts is reported. This parameter clearly illustrates the failure of reperfusion to restore adequate mechanical functions in the untreated hearts. In contrast, $F-1,6-P_2$-treated hearts were characterized by a good recovery of the heart rate.

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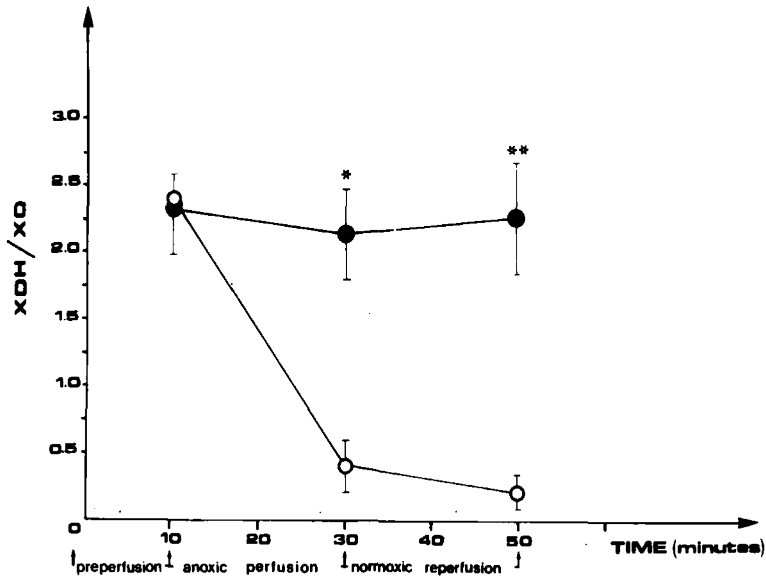

FIGURE 1 Conversion of XDH into XO after anoxia and reperfusion in control (○) and F-1,6-P_2-treated hearts (●) as expressed by the change in the XDH/XO ratio. Each point is the mean of 6 different hearts. Standard deviations are represented by vertical bars. *$p < 0.01$; **$p < 0.001$.

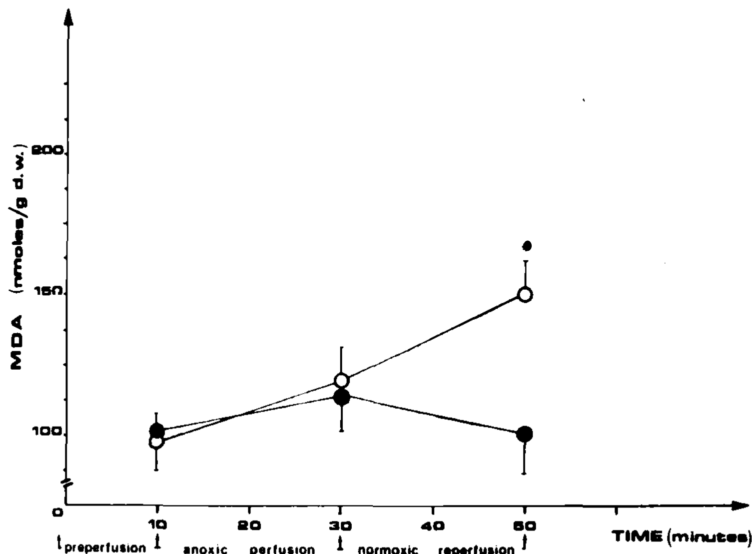

FIGURE 2 Concentrations of TBARS (as MDA equivalents) in control (○) and F-1,6-P_2-treated hearts (●) after anoxia and reperfusion. Each point is the mean of 6 different hearts. Standard deviations are represented by vertical bars. *$p < 0.001$.

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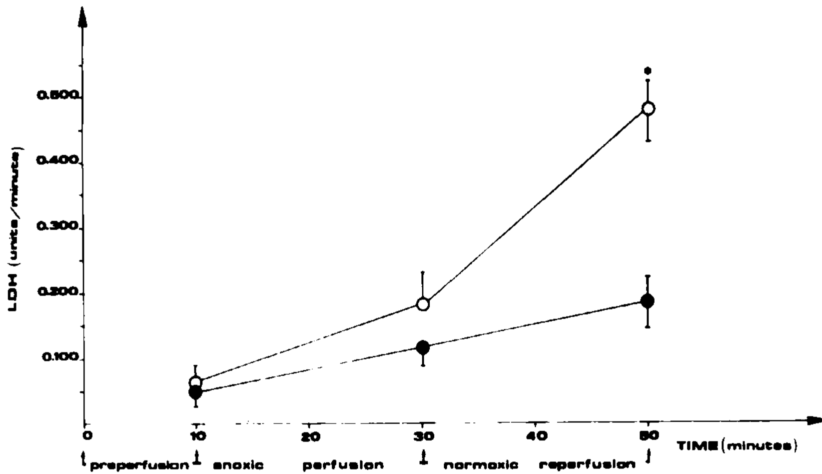

FIGURE 3 Lactate dehydrogenase activity in the perfusates of control (○) and F-1,6-P_2-treated hearts (●). Perfusates relative to each step were collected separately and subsequently assayed for LDH content. Each point is the mean of 6 perfusates referring to 6 different hearts. Standard deviations are represented by vertical bars. $*p < 0.001$.

DISCUSSION

The role of oxygen free-radicals in increasing the extent of damage to anoxic/ischaemic and reperfused tissues has been evident in the last few years.[26] Several reports have been published concerning the measurement of damage directly related to oxygen radicals in the reperfused intestine,[27] kidney,[28] liver[29] and brain.[30] However, data referring to anoxic/ischaemic and reperfused heart are still controversial and certainly unclear. For instance, a decrease of the concentration of GSH has been observed only after prolonged and very severe conditions of anoxic perfusion,[31,32] and irreversible conversion of XDH into XO has been reported by others to occur only after several hours of ischaemia.[33] Moreover, XDH seems to be localized within the endothelial cells of the vessels and not inside the myocytes.[11] This renders less clear the mechanism by which superoxide anions, produced externally to myocardial cells via the oxidation of purine molecules, might increase the damages of reperfused heart tissue. It could be also worth mentioning that xanthine oxidase is not present in several mammals,[34] thus complicating the hypothesis about the role of oxygen free-radicals in the mechanism of the oxygen paradox.

Nonetheless, reports describing transformation of XDH into XO,[5-7] increase of lipid peroxidation,[35-37] and indirect evidence of the involvement of oxygen-derived free-radicals in different models of reperfused heart, i.e. beneficial effects either of scavenging molecules,[38,39] or of specific inhibitors of XO,[40,41] have been reported by many authors; in this context, a direct observation (EPR) of the production of oxygen radicals by isolated bovine foetal aortic endothelial cells has recently been reported.[42]

In our experiments, in order to produce the optimal conditions for those molecular changes which lead to the irreversible conversion of XDH into XO in the myocardium, we subjected isolated Langendorff-perfused rat hearts to 20 minutes of substrate-free anoxic perfusion followed by 20 minutes of normoxic reperfusion. After 20

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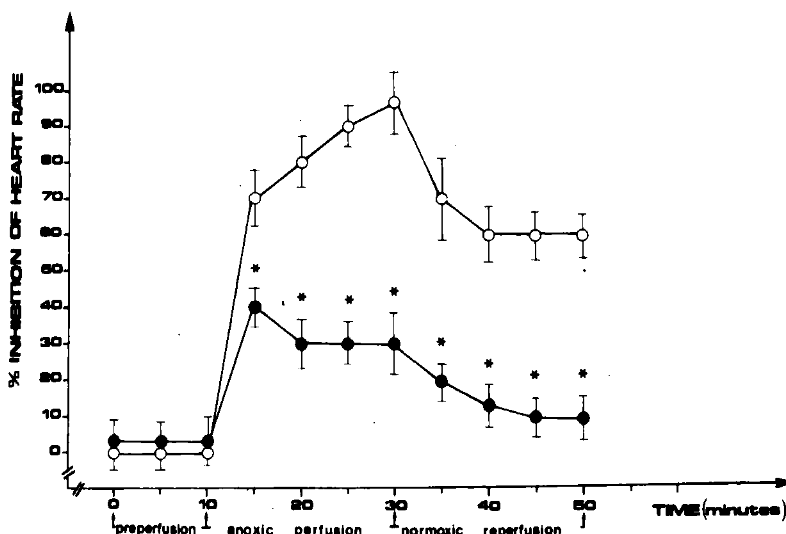

FIGURE 4 Inhibition of heart rate (expressed as the percent of initial) after anoxia and reperfusion in control (O) and F-1,6-P_2-treated hearts (●). Values are recorded once every 5 minutes from the beginning of the experiments. Each point is the mean of 6 different hearts. Standard deviations are represented by vertical bars. *$p < 0.001$.

minutes of anoxic perfusion we observed a 4 to 6 fold decrease in ATP and CrP respectively; this was followed by only a partial recovery after 20 minutes of normoxic reperfusion with a glucose-containing medium. Such a dramatic decrease of the phosphorylated compounds may be responsible for the irreversible transformation of XDH into XO as demonstrated by the 5 fold decrease of the XDH/XO ratio determined at the end of anoxia in the control hearts. In turn, the activation of this superoxide generating system could be related to the 50% increase in the TBARS content of control hearts recorded at the end of reoxygenation. This could be also linked to the 7 fold increase of LDH observed in the perfusates of control hearts at the same step. However, it cannot be excluded that the large amount of LDH released by reperfused hearts is, at least to a certain extent, due to the partial recovery of myocardial functions, i.e. a mechanical disruption of anoxia-damaged myocytes.

Data obtained by perfusing the heart with 5 mM F-1,6-P_2 throughout the different steps of perfusion (preperfusion, anoxic perfusion and normoxic reperfusion) give, in our opinion, two major indications: i) the maintainance of adequate concentrations of high-energy metabolites inhibits the conversion of XDH into XO thereby avoiding the oxygen radical-induced production of TBARS; ii) exogenous F-1,6-P_2 is a favourable metabolic substrate for the heart.

In fact, F-1,6-P_2-treated hearts showed, after anoxia, higher concentrations both of ATP and CrP compared to control hearts, albeit both of them were 50% lower than the initial value. However, the XDH/XO ratio in F-1,6-P_2-perfused hearts was unchanged either at the end of anoxia or reperfusion thereby suggesting that there might exist a threshold of ATP concentration beyond which the irreversible conversion of XDH into XO is triggered. Furthermore, data concerning the XDH/XO ratio and TBARS for both control and F-1,6-P_2-treated hearts seem to indicate a close relationship between these two parameters.

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In addition, F-1,6-P_2 induced an almost complete recovery of ATP and CrP concentrations after reperfusion and decreased LDH release both after anoxia and reperfusion. It is worth noting that the difference in the LDH release by the two groups of hearts during reperfusion might be attributed in part to the oxygen free-radicals generated in control hearts by the XO-activated system.

On the basis of the differences observed for all the parameters examined between control and F-1,6-P_2-treated hearts it may be hypothesized that F-1,6-P_2 elicits its effects by acting as an energetically-favourable substrate. This hypothesis is supported by two considerations: i) the highly significant differences observed at the end of anoxia with respect to control hearts can be explained only by considering that a certain amount of the F-1,6-P_2 has been taken up by the myocytes and metabolized to produce ATP (4 moles of ATP per mole of F-1,6-P_2 consumed are produced by glycolysis). ii) the beneficial effects of F-1,6-P_2 should be ascribed to the molecule itself and not to its degradation products, such as fructose, since it has been demonstrated that the heart is not able to utilize fructose for its energy demand.[43]

It therefore seems that F-1,6-P_2 administration greatly reduces anoxia and reperfusion injury and is, consequently, a potentially useful drug for the pharmacological treatment of acute myocardial infarction. Moreover, these results show that stimulation of glycolysis may contribute to a reduction of anoxia (ischaemia) and reperfusion injuries in the heart.[44-47]

Further studies to evaluate the susceptibility of heart tissue to oxidative stress and to elucidate fully the mechanism of action of F-1,6-P_2 are needed.

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