

Hypothermic preservation of rat hearts using antifreeze glycoprotein

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Short title: Hypothermic heart preservation with AFGP

Summary

Background

Antifreeze proteins are an effective additive for low-temperature preservation of solid organs. Here, we compared static hypothermic preservation with and without antifreeze glycoprotein (AFGP), followed by nonfreezing cryopreservation of rat hearts.

Methods

The heart was surgically extracted and immersed in one of the cardioplegia solutions after cardiac arrest. Control rat hearts (n = 6) were immersed in University of Wisconsin (UW) solution whereas AFGP-treated hearts (AFGP group) (n = 6) were immersed in UW solution containing 500 µg/mL AFGP. After static hypothermic preservation, a Langendorff apparatus was used to reperfuse the coronary arteries with oxygenated Krebs-Henseleit solution. After 30, 60, 90, and 120 min, the heart rate (HR), coronary flow (CF), cardiac contractile force (max dP/dt), and cardiac diastolic force (min dP/dt) were measured. Tissue water content (TWC) and tissue adenosine triphosphate (ATP) levels in the reperfused preserved hearts were also assessed. All the parameters were compared between the control and AFGP groups.

Results

Compared with the control group, the AFGP group had significantly ($p < 0.05$) higher values of the following parameters: HR at 60, 90, and 120 min; CF at all four time

points; max dP/dt at 90 min; min dP/dt at 90 and 120 min; and tissue ATP levels at 120 min. TWC did not differ significantly between the groups.

Conclusions

The higher HR, CF, max dP/dt, min dP/dt, and tissue ATP levels in the AFGP compared with those in control hearts suggested that AFGP conferred superior hemodynamic and metabolic functions. Thus, AFGP might be a useful additive for the static/nonfreezing hypothermic preservation of hearts.

Key words: antifreeze glycoprotein, hypothermic preservation, rat

Introduction

Heart transplantation is an established treatment for end-stage heart failure. Methods used to preserve donor hearts are broadly classified as coronary perfusion preservation and static hypothermic preservation. Coronary perfusion is effective in extending the ischemic time, but requires specialized circuits and devices (Michel *et al.* 2015). Thus, owing to its ease of use and low cost, static hypothermic preservation has become the standard method for heart preservation in cases of heart transplantation.

Static hypothermic preservation of organs reduces metabolic processes of the preserved organ and is thought to be effective for preservation at lower temperatures (Belzer and Southard 1988). However, cryopreservation methods can lead to low-temperature injuries, such as electrolyte imbalances and cellular edema (Jahania *et al.* 1999). With constant improvements in the therapeutic outcomes of heart transplantations, reducing the preservation time of the donor heart is considered very important because, when the preservation time of the donor heart exceeds 200 min, the long-term outcome is poor (Stehlik *et al.* 2011). Two problems associated with static hypothermic preservation are low-temperature injury and time constraints. Approaches to overcome these problems would make it easier to transport donor hearts over long distances.

In 1969, antifreeze proteins (AFPs) were discovered in fish of the Nototheniidae family, which are native to the Antarctic Ocean. In the low temperatures of the Antarctic Ocean, fish are constantly in danger of their blood freezing. However, the AFPs produced in their bodies lower the freezing point of their blood and inhibit the growth of ice crystals. AFPs are classified as AFPs I–IV and antifreeze glycoproteins (AFGPs) (Harding *et al.* 2003). Besides their ability to lower the freezing point of body fluids, other effects of AFPs in hypothermic environments include cell preservation via interruption of cell membrane ion channels, and stabilization of the cell membrane (Rubinsky *et al.* 1990) (Rubinsky *et al.* 1992).

In recent years, studies on large-scale purification of AFPs have been carried out, pioneering their practical applications (Nishimiya *et al.* 2008). As a result, the focus of research has expanded to include high-purity refinement of AFGP (Burcham *et al.* 2006). It has further been reported that AFGP is effective as a cryoprotectant for the hypothermic storage of islet and sperm cells (Matsumoto *et al.* 2006, Qadeer *et al.* 2015). However, these reports have focused on cell preservation. Thus, the effectiveness of AFGP as a cryoprotectant for solid organ storage has yet to be established. In the present study, we evaluated the effectiveness of static/nonfreezing hypothermic preservation of rat hearts in a preservative solution with added AFGP based on our hypothesis that AFGP would be useful as a cryoprotectant in a static

hypothermic preservation solution used in the hypothermic preservation of cardiomyocytes.

Methods

The methods for removing rat hearts, reperfusion experiments using the Langendorff apparatus, and for measuring cardiac function are described below and were the same as reported by us earlier for an isolated rat heart model (Kato *et al.* 2012). Briefly, we used adult Wistar male rats (age: 10–12 weeks, body weight: 250–300 g; Japan SCL, Shizuoka, Japan). Experimental animals were handled in accordance with the “Guide for the Care and Use of Laboratory Animals: Eighth Edition” of the Institute for Laboratory Animal Research (2010). Approval was obtained from the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-153592). Animals were anesthetized initially by making them inhale 4%–5% isoflurane (Wako Pure Chemical Industries, Osaka, Japan), which was then switched to maintenance levels of 1.5%–3%. The anterior region of the animals’ necks, and the tracheas were incised longitudinally. After tracheal intubation, the animals were placed on mechanical ventilators (initial settings: 1 Hz; tidal volume, 3.2 mL; and fraction of inspired oxygen, 0.21). Laparotomy and thoracotomy were performed, and the heart, inferior vena cava, superior vena cava, and ascending aorta were exposed.

Five hundred units of heparin (Mochida Pharmaceutical, Tokyo, Japan) were injected via the inferior vena cava, and both the inferior and superior vena cava were dissected. The ascending aorta was also dissected and a 2-mm cannula for administration of the cardioplegia solution was connected (Medtronic, Minneapolis, MN, USA). The cardioplegia solution (4 °C; total, 50 mL; injection rate, 5 mL/min) was administered via this cannula, and the heart was stopped. The heart was surgically extracted and immersed in one of the cardioplegia solutions described below. In the control group, the University of Wisconsin (UW) cardioplegia solution was used (Viaspan [in g/L: pentafraction, 50; lactobionic acid, 35.83; potassium phosphate monobasic, 3.4; magnesium sulfate heptahydrate, 1.23; raffinose pentahydrate, 17.83; adenosine, 1.34; allopurinol, 0.136; total glutathione, 0.922; potassium hydroxide, 5.61; pH 7.4]; Astellas Pharma, Tokyo, Japan). In the AFGP group, the cardioplegia solution used was the UW solution containing 500 µg/mL AFGP. The preservative immersion solutions used were the same as the cardioplegia solutions; that is, the UW solution in the control group and the UW solution with AFGP 500 µg/mL in the AFGP group.

AFGP

The AFGP used in these experiments was extracted from fish in the cod family (Nichirei Foods, Chiba, Japan). AFGP, which is a solid, is a highly refined product guaranteed to have a purity of at least 90%. It was dissolved in the UW solution to 500

µg/mL, and this solution was allowed to stand for 2 h to remove bubbles before use.

Experimental groups

Cardioplegia was performed under two conditions. In the control group (n = 6), for which the conventional method was used, immersion preservation was performed in the UW solution. In the AFGP group (n = 6), immersion preservation was performed in the UW solution + AFGP 500 µg/mL. In both the groups, immersion was carried out at 4 °C for 24 h.

After preservation for 24 h, hearts were connected to a Langendorff apparatus and reperfusion, consisting of retrograde perfusion from the ascending aorta to the coronary arteries, was performed (Fig. 1). The perfusion solution was infused into the coronary arteries at a pressure of 75 mmHg for 120 min. The perfusion was performed using the Krebs-Henseleit solution (in mM: NaCl, 118; KCl, 4.7; MgSO₄·7H₂O, 1.2; CaCl₂·2H₂O, 2.5; NaHCO₃, 25; glucose, 11.0; KH₂PO₄, 1.2; pH = 7.6), which was made at our facility. This solution was heated to 37.0 °C and buffered with 95% oxygen and 5% carbon dioxide.

Functional measurements in the graft

Cardiac function measurements performed in conjunction with the reperfusion experiments were as follows: Left ventricular pressure was measured by connecting a pressure transducer (VO1706TSPL03; Edwards Life Science, Irvine, CA, USA) to a

3Fr latex balloon catheter (Unique Medical, Komae-shi, Tokyo, Japan) filled with physiological saline, which was inserted into the left ventricle via the left atrium. The latex balloon catheter was then inflated to a diastolic pressure of 8 mmHg. Pressure changes were analyzed using LabChart7 version 7.3.7 (AD Instruments, Bella Vista, New South Wales, Australia). At 30, 60, 90, and 120 min after the start of reperfusion, heart rate (HR), coronary flow (CF), max dP/dt (rate of pressure change), and min dP/dt (negative rate of pressure change) were measured. CF was calculated on the basis of the amount of coronary perfusion solution used. Max dP/dt indicates cardiac contractile force and min dP/dt indicates cardiac diastolic force (Kato *et al.* 2012). During cardiac function measurements, pacing was not used to maintain the heartbeat; instead, the hearts were allowed to beat spontaneously.

Tissue ATP levels

The extraction of ATP was performed according to the method reported previously (Prokudina *et al.* 2019). After 120 min of reperfusion, approximately 0.1 g of the myocardium was removed surgically from the beating hearts. This portion of the myocardium was snap frozen and homogenized in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). The weight of the frozen myocardium sample was measured and 1 mL of protein removal solution (perchloric acid, 0.6 N) per 0.1 g of myocardium tissue was added. The homogenate was centrifuged at $4\text{ }^{\circ}\text{C}/3,000\text{ g}$ for 10 min. The supernatant was then

neutralized in a triethanolamine/potassium carbonate solution and preserved at -80 °C until ATP measurements were taken. The concentration of ATP was measured by absorption spectroscopy (Lucifell 250 Plus; Kikkoman, Tokyo, Japan). The ATP levels are expressed in μ mole per dried tissue weight of left ventricular tissue. To reduce ATP consumption to a minimum during sample preparation, the collection site was the left ventricular apex, which allowed samples to be collected quickly and easily while the heart was beating.

Tissue water content (TWC)

After 120 min of reperfusion, the heart tissue remaining after removal of the portion used for ATP extraction was air-dried over a 72 h period in the laboratory, where the environment was controlled at a room temperature of 23 °C and humidity of 30%. The percentage TWC was calculated using the following formula: $TWC = (WW - DW)/WW \times 100$, where WW was the “wet tissue weight” (g) and DW was the “dry tissue weight” (g).

Statistical analyses

Data are presented as means \pm standard deviation and median (interquartile range) values, and are plotted on graphs. The statistical software used was EZR version 1.38 (Kanda *et al.* 2013). The control and AFGP groups were compared for the following parameters: HR, CF, max dP/dt, min dP/dt, tissue ATP level, and TWC. All measured

values were determined to be either in normal or non-normal distributions using the Shapiro-Wilk normality test. When sampling data for both the control and AFGP groups were normally distributed, Student's *t*-test was performed. In cases where data were non-normally distributed, the Mann-Whitney U test was applied. Significance was set at a p-value < 0.05.

Results

Heart rate

Comparisons of the control versus AFGP group HR indicated the following values at 30, 60, 90, and 120 min: 80.7 ± 1.4 vs. 80.0 ± 2.5 /min ($p = 0.55$), 78.7 ± 37.6 vs. 134 ± 24.3 /min ($p = 0.013$), 98.5 ± 38.4 vs. 167 ± 33.1 /min ($p = 0.0079$), and 103 ± 46.7 vs. 174 ± 33.0 /min ($p = 0.012$), respectively (Fig. 2). Thus, at 60, 90, and 120 min after the start of the reperfusion experiments, the AFGP group had significantly higher autonomous HRs than the control group.

CF

Comparisons of the control versus AFGP group CF indicated the following values at 30, 60, 90, and 120 min: 2.47 ± 1.93 vs. 4.60 ± 0.59 mL/min ($p = 0.028$), 2.03 ± 1.27 vs. 4.87 ± 0.39 mL/min ($p = 0.0047$), 2.92 ± 1.44 vs. 5.37 ± 0.39 mL/min ($p = 0.0024$), and 3.50 ± 1.83 vs. 5.77 ± 0.53 mL/min ($p = 0.016$), respectively (Fig. 3). Thus, at all

the studied time points, the AFGP group had significantly higher CFs than the control group.

max dP/dt

Max dP/dt values of the control versus AFGP group at 30, 60, 90, and 120 min were as follows: 165 ± 128 vs. 463 ± 319 mmHg/s ($p = 0.060$), 187 ± 91.1 vs. 397 ± 300 mmHg/s ($p = 0.13$), 127 ± 66.6 vs. 488 ± 236 mmHg/s ($p = 0.0048$), and 177 ± 129 vs. 521 ± 370 mmHg/s ($p = 0.056$), respectively (Fig. 4A). At 90 min after the start of reperfusion, the max dP/dt was significantly higher in the AFGP group than in the control group. At other time points, the contractile force of the preserved hearts in the AFGP group was not significantly higher.

min dP/dt

Comparisons of the control versus AFGP group for min dP/dt at 30, 60, 90, and 120 min were as follows: 170 ± 143 vs. 435 ± 298 mmHg/s ($p = 0.078$), 177 ± 87.3 vs. 350 ± 232 ($p = 0.12$), 127 ± 65.6 vs. 461 ± 235 mmHg/s ($p = 0.0072$), and 159 ± 90.6 vs. 532 ± 384 mmHg/s ($p = 0.044$), respectively (Fig. 4B). At 90 and 120 min after the start of reperfusion, the min dP/dt in the AFGP group was significantly higher than that in the control group. At other time points, the diastolic force of the preserved hearts in the AFGP group was not significantly higher.

TWC

The TWC was $80.7\% \pm 1.4\%$ vs. $80.0\% \pm 2.5\%$ ($p = 0.55$) for the control and AFGP groups, respectively (Fig. 5A). This indicated that there was no significant difference in the myocardial edema of post-reperfusion preserved hearts.

ATP levels

Tissue ATP levels were 1.10 ± 0.30 vs. 4.28 ± 2.34 $\mu\text{mol/g}$ (dry weight) ($p = 0.0079$) for the control and AFGP groups, respectively (Fig. 5B). This indicated that the preserved hearts in the AFGP group maintained higher levels of ATP in their tissues compared to the control group.

Discussion

In this study, we added purified AFGP to the immersion solution used for cryopreserving rat hearts at $4\text{ }^{\circ}\text{C}$ for 24 h. In our reperfusion experiments conducted on the preserved hearts, we measured HR, CF, max dP/dt, and min dP/dt as indicators of their hemodynamic functions. The HR at all time points after 30 min following the start of reperfusion (60, 90, 120 min); CF at all time points following the start of reperfusion; and both max dP/dt and min dP/dt at 30, 90, and 120 min following the start of reperfusion were higher in the AFGP group than in the control group. These data showed that the preserved hearts treated with AFGP maintained better hemodynamic function than those in the control group. We also measured tissue ATP

levels at the end of reperfusion to assess metabolic function. The results showed higher values in the AFGP group, indicating that AFGP helped maintain metabolic functions.

In recent years, it has become possible to prolong the ischemia time through the perfusion of preservation solution into solid organs, such as the heart and kidney. However, specialized circuits and apparatus are required to maintain electrolytes, oxygen saturation, and perfusion pressure of the perfusion solution (Michel *et al.* 2015). Thus, static hypothermic preservation has become the main method for preserving hearts owing to its ease and low cost. Because the metabolic processes of preserved organs are reduced when they are stored using static hypothermic preservation, this method is useful in preservation at low temperatures (Belzer *et al.* 1988). However, electrolyte imbalances and cell edema are limitations associated with static hypothermic preservation (Rubinsky *et al.* 2003).

Currently, static hypothermic preservation of hearts for use in heart transplantation is generally performed at 4 °C, and the safe time limit is between 4 and 6 h (Jahania *et al.* 1999, George *et al.* 2011). Attempts have been made to develop stable hypothermic preservation methods that resolve these problems, such as mixing cryoprotectants like glycerol into the immersion solution (Yang *et al.* 1993, Sakaguchi *et al.* 1998). In addition, although the development of a variety of organ preservative solutions with

adjusted osmotic pressure and electrolytes is ongoing, these have yet to achieve an extension of the ischemic time close to that of static hypothermic preservation in heart transplantation. Thus, we believe that the discovery of a new cryoprotectant that is useful in hypothermic preservation would help increase the time-related safety limit of static hypothermic preservation.

Biological substances identified in Nototheniidae fish, which are native to cold regions of the world, lower the freezing point of body fluids and restrict the growth of ice crystals (DeVries and Wohlschlag 1969). One of these biological substances that has been identified is AFP. Fish-derived AFPs are classified as AFP I–IV and AFGP. Each AFGP consists of a number of repeating tripeptide units of (Ala-Ala-Thr)_n, with minor sequence variations. The glycoproteins isolated from the Nototheniidae fish have been further classified as AFGP1–8 on the basis of their relative rates of electrophoretic migration. The number of tripeptide repetitions varies, and the molecular weight of AFGP is between 2600 and 33,000 Da (Harding *et al.* 2003). In addition to their ability to lower the freezing point, AFPs protect cells through the transfer of electrolytes via the cell membrane and prevent cell edema by forming a protective layer around the cell membrane when cells are preserved in hypothermic environments (Rubinsky *et al.* 1990, Rubinsky *et al.* 1992). Tomczak *et al.* proposed that AFGPs may form a monolayer covering the membrane surface, thereby, reducing the leakage of ions

across the membrane as it is cooled through its thermal transition temperature (Tomczak *et al.* 2002).

In recent years, hypothermic preservation experiments using pancreas cells, sperm, and oocytes have focused on the cell-protective properties of AFPs, and it has been reported that they are useful as alternative cryoprotectants to dimethyl sulfoxide and glycerol (Matsumoto *et al.* 2006, Qadeer *et al.* 2015, Rubinsky *et al.* 1991, Kamijima *et al.* 2013, Prathalingam *et al.* 2006). Recent studies have focused on practical applications of AFPs as substrate additives for the hypothermic preservation of solid organs. AFPI has been used as a substrate additive in the hypothermic preservation of livers (Soltys *et al.* 2001). In addition, experiments using AFPI and AFPIII as cryoprotectants for the hypothermic preservation of hearts have reported that cardiac functions following reperfusion are preserved satisfactorily, and that structures of the myocardium and mitochondria remain intact (Amir *et al.* 2004, Amir *et al.* 2005). However, in all the studies indicating that AFPs are useful in the hypothermic preservation of solid organs, AFP has been used; thus, it remains unknown whether AFGP is useful in the preservation of solid organs (Wang *et al.* 1994, Mugnano *et al.* 1995).

In recent years, studies on the large-scale refinement of AFPs have been performed, which have led to practical applications of these compounds (Nishimiya *et al.* 2008).

The focus of research has expanded to include the high-purity refinement of AFGP (Burcham *et al.* 1986). In the present study, we focused on AFGP and carried out experiments on the static/nonfreezing hypothermic preservation of rat hearts in preservative solution with added AFGP. This was based on our hypothesis that AFGP would be useful as a cryoprotectant in the immersion solution used in the hypothermic preservation of solid organs by preserving the hemodynamic and metabolic functions of the heart. The results showed that post-reperfusion tissue ATP levels were significantly higher in the AFGP group than in the control group. There was a correlation between cardiac function and ATP levels in the myocardial tissue; the latter is a good indicator of the status of preserved hearts (Stringham *et al.* 1992, Pernot *et al.* 1983, Sakaguchi *et al.* 1996). Specifically, high tissue ATP levels indicate an excellent status of the cardiomyocytes, even in terms of metabolic function.

We measured TWC as an indicator of myocardial edema during static hypothermic preservation as it allows an assessment of damage to the preserved heart (Kato *et al.* 2012). We found that, in contrast to the satisfactory hemodynamic and metabolic functions observed in preserved hearts in the AFGP group, TWC in the AFGP group was low, although not significantly. However, research has shown that myocardial edema may increase despite good cardiac functions observed in post-preservation reperfusion experiments; this is believed to indicate the complexity of the action of

hydrostatic pressure on the myocardium (Zhang *et al.* 2010). Because we found that CF was significantly higher in the AFGP group than in the control group, it is possible that higher amounts of perfusion solution penetrated cardiomyocytes and stroma in the AFGP group via the coronary arterial endothelia. This, in turn, may have increased the hydrostatic pressure in those locations. Furthermore, it is conceivable that in cells and organoids in preservation experiments wherein AFGP was used as a substrate additive, AFGP may have been localized to cell membrane surfaces that were in contact with the immersion solution (Tomczak *et al.* 2002, Huelsz-Prince *et al.* 2019). Although we did not assess the localization of AFGP in this study, we believe that it may act primarily on the coronary artery endothelium and endocardial cells of the preserved heart that are in direct contact with the immersion solution. The degree of edema observed in a large portion of cardiomyocytes that had no direct contact with the immersion solution was the same in preserved hearts both in the control and AFGP groups. This might explain the similarity between the control and AFGP groups in terms of TWC.

Some studies on the use of AFGP to preserve cells have used approximately 10 mg/mL of the protein (Sakaguchi *et al.* 1998, Kamijima *et al.* 2013, Huelsz-Prince *et al.* 2019, Hirano *et al.* 2008). However, AFGP increases the viscosity of solutions in a concentration-dependent (1–10 mg/mL) manner (Eto and Rubinsky 1993). When

dissolving AFGP in the UW solution at the preliminary experiment stage, the solution was highly viscous and it was difficult to remove bubbles. In addition, the high viscosity of the UW solution itself might explain endovascular endothelial damage during coronary perfusion (Mankad *et al.* 1992). From the perspective of the coronary vascular bed, we believe it is important to prevent increased viscosity of the immersion solution. One study that reported the use of low AFGP concentrations found that 500 µg/mL was effective in protecting pancreatic cells during cryopreservation (Matsumoto *et al.* 2006). Therefore, we used a relatively low concentration of 500 µg/mL in all our experiments.

Our study had some limitations. First, the model was *in vitro* with preserved hearts connected to a Langendorff coronary perfusion apparatus and the use of Krebs-Henseleit perfusion solution. Second, the safety of using AFGP during the perfusion of blood containing white blood cells and cytokines is unknown. This warrants the need for additional studies to verify that AFGP can be used safely in the preservation of human organs. Third, an *in vivo* model using allogenic transplantation in rats must be performed to investigate the usefulness of AFGP. Finally, because we did not verify the localization of AFGP and the sites where it is effective, the need to further study the importance of AFGP localization remains. Overall, there have been few studies on cryopreservation using AFGP. Thus, the optimum conditions for its use, including the

optimum concentration and temperature range, need to be determined and established.

This is an innovative report applying AFGP to the preservation of a solid organ. In this study, we used AFGP as a substrate additive in the static/nonfreezing hypothermic preservation of rat hearts. In the AFGP group, hemodynamic and metabolic functions were better maintained in the preserved hearts than in the control group. The results are expected to extend the allowable ischemic time of organ transplantation and permit the transport of transplant organs over long distances easily.

Conflicts of Interest

Shintaro Takago, Isao Matsumoto, and Hiroki Kato received AFGP from Nichirei Foods Inc. (Chiba, Japan). This work was supported by Japanese Society for the Promotion of Science, Grant/Award Number: JSPS KAKENHI Grant Number JP19K18174.

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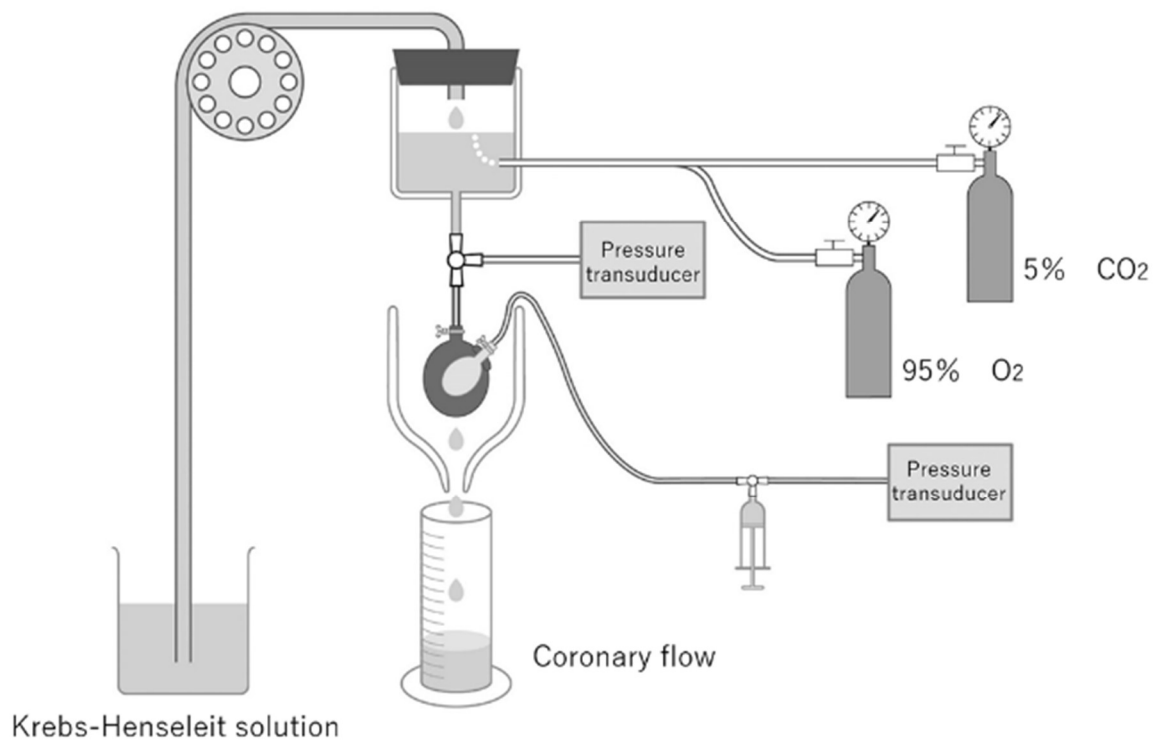


Fig 1. Reperfusion experiments using the Langendorff apparatus.

The perfusion solution was infused into the coronary arteries at a pressure of 75 mmHg for 120 min. Krebs–Henseleit solution buffered with 95% oxygen and 5% carbon dioxide. Left ventricular pressure was measured by connecting a pressure transducer to a 3Fr latex balloon catheter, which was inserted into the left ventricle via the left atrium.

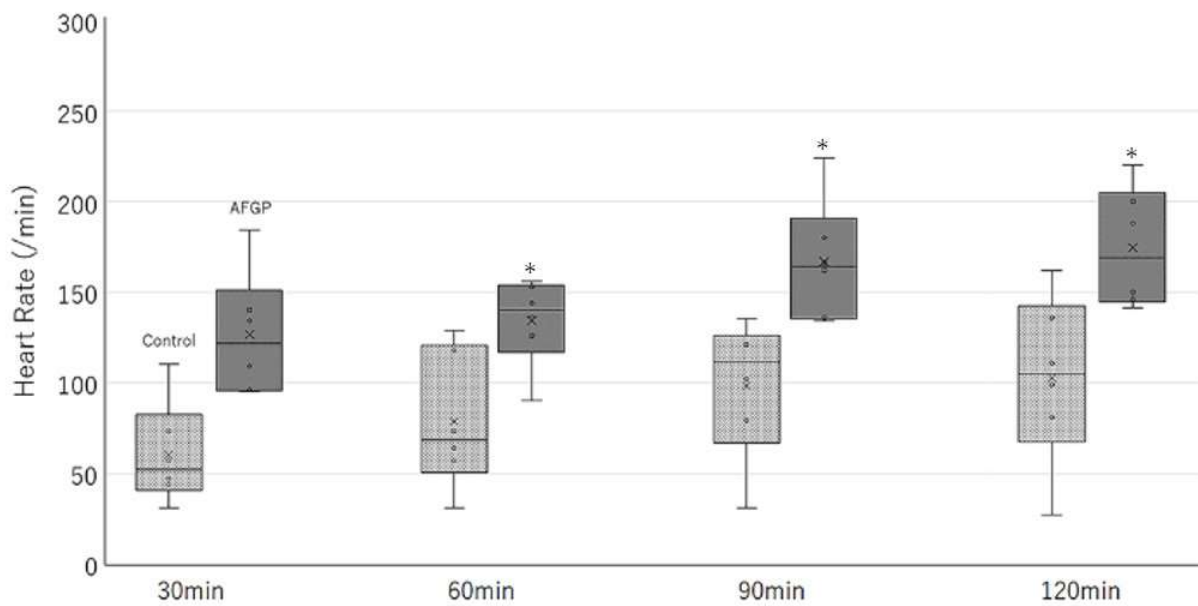


Fig 2. Heart rate during reperfusion.

The AFGP group had significantly higher HRs than the control group at 60, 90, and 120 min after the start of the reperfusion experiments (*p<0.05).

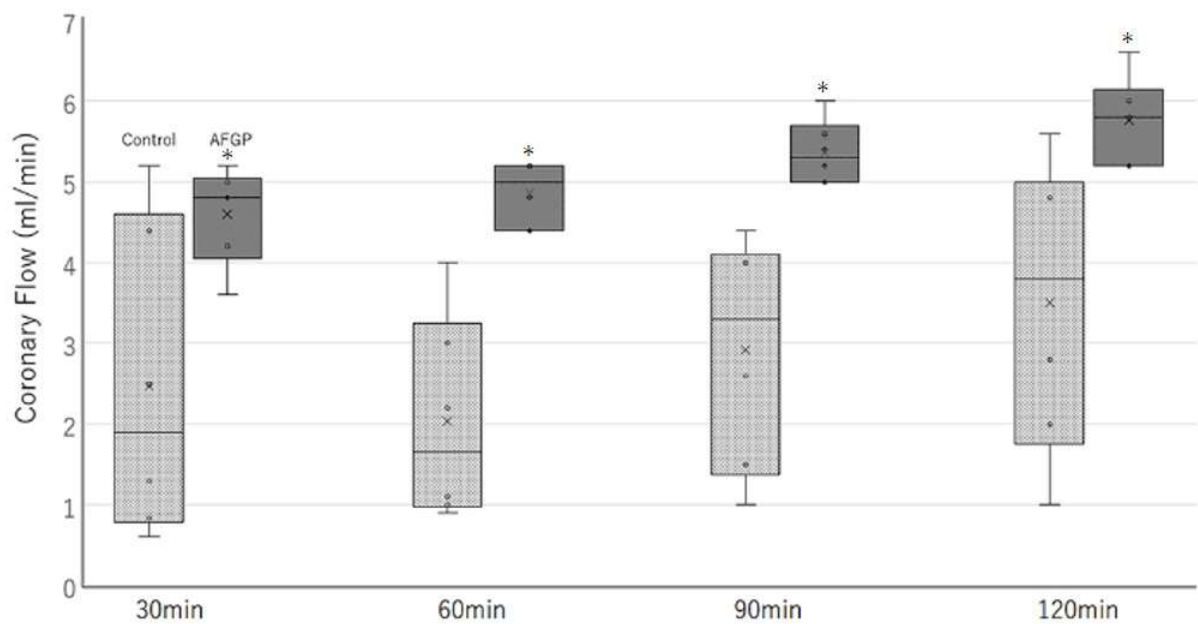


Fig 3. Coronary flow during reperfusion.

The AFGP group had significantly higher CFs than the control group at all studied time points (*p<0.05).

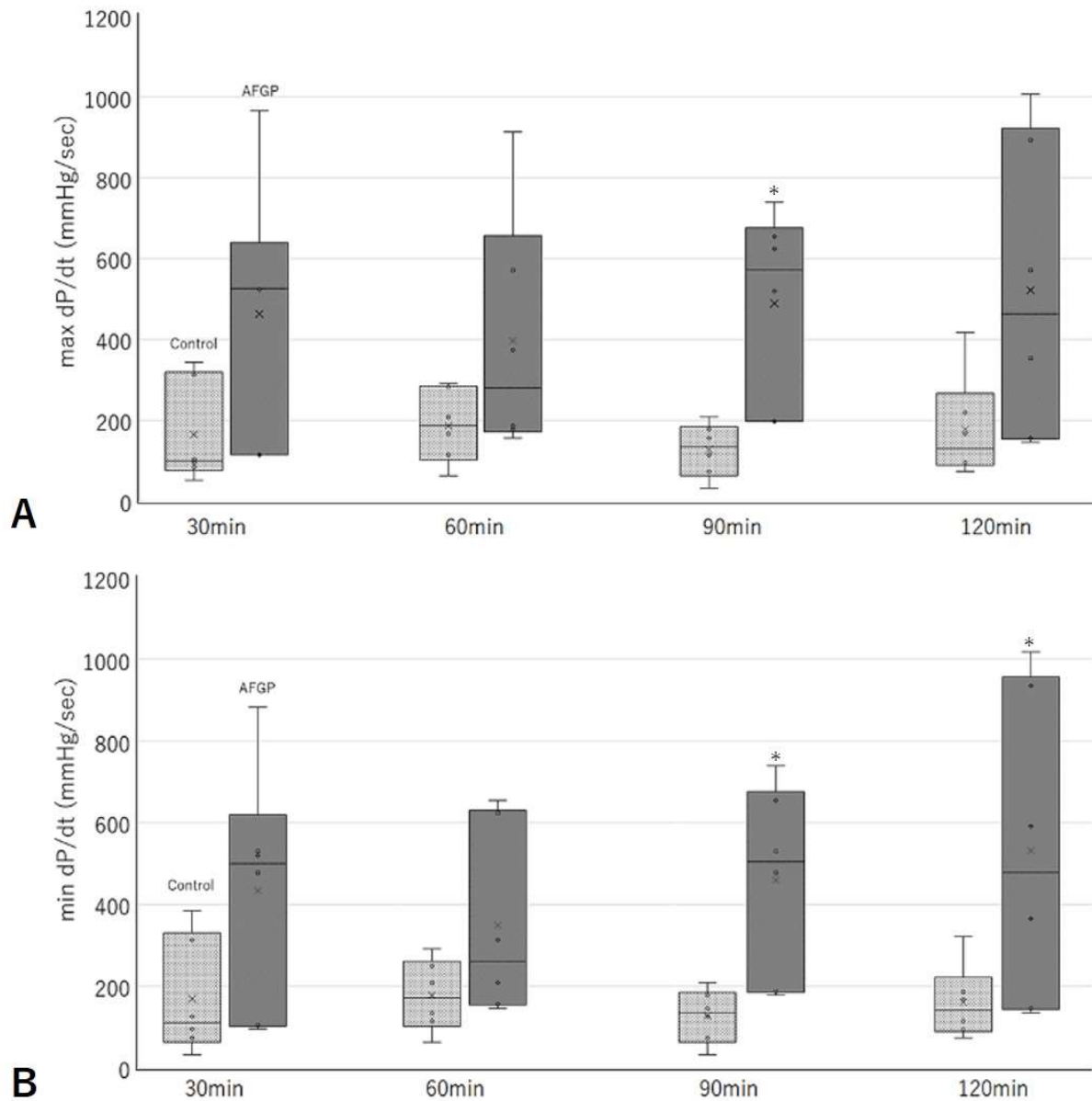


Fig 4A. max dP/dt (rate of pressure change) flow during reperfusion.

The AFGP group had significantly higher max dP/dt than the control group at 90min after the start of the reperfusion experiments (*p<0.05).

Fig 4B. min dP/dt (negative rate of pressure change) flow during reperfusion.

The AFGP group had significantly higher min dP/dt than the control group at 90 and 120 min after the start of the reperfusion experiments (*p<0.05).

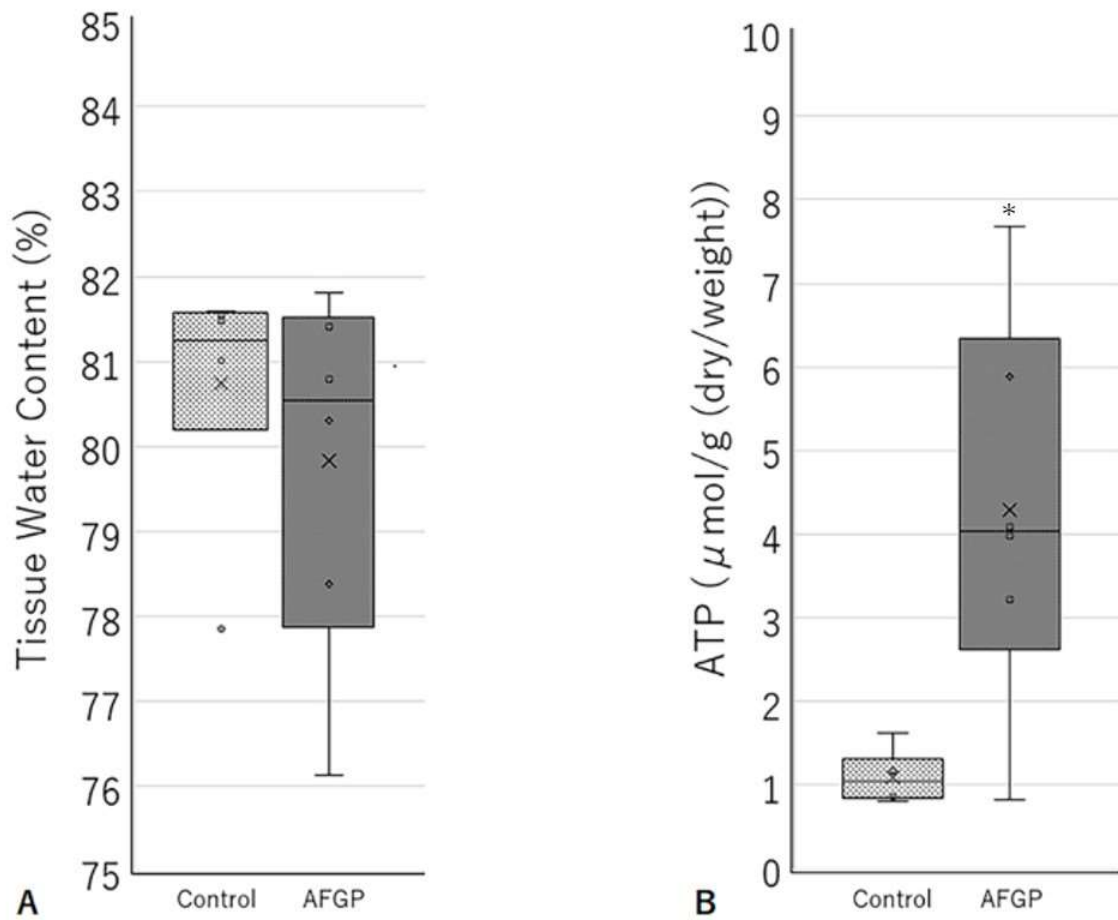


Fig 5A. Tissue water content (TWC) after 120 min of reperfusion. TWC did not differ significantly between two groups.

Fig 5B. Tissue adenosine triphosphate (ATP) levels after 120 min of reperfusion. The AFGP group maintained higher levels of ATP in their tissues compared to the control group (* $p < 0.05$).