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*Original article*

# Oxidative parameters and expression of 70kDa heat shock proteins in pig heart tissue after transport and slaughter

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## Abstract

In view of the significant role of Hsp70 in protecting the organism against the destructive effects of stress, and the possibility of using this protein as a marker of the infarction process in the heart, the aim of this study was to conduct an evaluation of the expression of 70kDa heat shock proteins (Hsp70) and the concentration of TBARS (thiobarbituric acid reactive substances) and nitric oxide ions (NO), determined as nitrite ions, as markers of oxidative stress in hearts obtained from healthy pigs following slaughter and pigs which had died during or immediately after transport with symptoms of sudden cardiac death.

The material consisted of hearts obtained from 90 pigs following slaughter and from pigs which had died. Oxidative stress was determined in heart lysates based on the concentration of TBARS and nitrite ions. Expression and concentration of Hsp70 were determined using SDS-PAGE, Western blotting, and ELISA.

Expression of Hsp70 was observed in hearts lysates obtained from slaughtered pigs and from those which had died with symptoms of sudden death. The strongest reaction in the Western Blotting was noted in hearts lysates from pigs with no pathological changes. The highest TBARS concentration was observed in lysates from hearts in pigs which had died during or immediately after transport. The highest concentration of NO ions, determined as nitrite ions, was noted in hearts from pigs with myocardial infarction lesions. The significant decrease observed in Hsp70 concentration in heart tissue obtained from the pigs which had died in comparison to the hearts from healthy pigs indicates the important role of this protein in protecting the heart muscle against the destructive effects of stress, which limits the occurrence of post-stress cardiomyopathy in pigs following transport.

**Key words**: Hsp70, oxidative stress, transportation, pigs, slaughter, welfare

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## Introduction

Transport and slaughter of animals is an important factor inducing a stress reaction and thereby reducing the animal well-being. In response to stress and the associated increase in activity of cortisol, corticosterone, and enzymes participating in oxidative processes in the organism, the production and release of heat shock proteins (Hsp) by cells increases (Urban-Chmiel et al. 2009, Minka et al. 2010). The functions of Hsp are mainly associated with the stabilization and regeneration of intracellular proteins and with controlling the immune activity of the cells of the organism. Increased production of these proteins is correlated with increased resistance of tissues and organs to damage induced by factors including ischaemic disease, free radicals, external toxic substances, and infectious agents. The presence of Hsp also plays an important role in controlling the spread of the inflammatory process (Bao et al. 2008). An increased Hsp70 level in the cells of the heart muscle can also be a useful marker of the transplant rejection process, due in part to the direct relationship between the level of this protein and the development of the transplant rejection process (Snoeckx et al. 2001).

Among farm animals, pigs are the most susceptible to stress factors associated with transport; in many cases irreversible changes occur within the organism, manifested in post-stress syndromes such as PSE (pale soft exudative) meat, DFD (dark firm dry) meat, and sudden cardiac death (Watanabe et al. 1996, Vecerek et al. 2006, Miranda-De La Lama et al. 2012). These disorders have a decisive role in reducing the quality of the products obtained. A particularly unfavourable phenomenon in pigs following transport is sudden death resulting from myocardial infarction (Geverink et al. 1998).

Such health problems are the consequence of metabolic disorders resulting from oxygen deficiency, electrolyte imbalance, and the development of oxidative stress, manifestations of which include increased intensity of lipid peroxidation processes, increased release of superoxide anions, and reduced antioxidant status (Wernicki et al. 2006, Breinekova et al. 2007). These changes result in disturbances in the production and release of lipid peroxidation final products (TBARS) and nitrogen ions (NO), and are caused by increased activation of myeloperoxidase and eosinophil peroxidase, enzymes involved in the inflammatory process and ischaemic changes (Baker et al. 2002). The stress-induced increase in the production and release of Hsp in pigs may play a significant role in limiting damage and death in transported animals, which can be a useful indicator of the welfare of pigs intended for slaughter. It has been demonstrated (Feder and Hofmann, 1999) that production and release of heat shock proteins, whose functions include protecting intracellular structures from damage (Bao et al. 2008, Zhang et al. 2012), increase in pigs during transport. The role of Hsp in reducing ischaemia associated with degradation of lipid membranes is not fully known, although many observations confirm that stability of cell membranes increases due to reduced activity of arachidic acid – a marker of phospholipid degradation (Van der Vusse et al. 1998).

In view of the significant role of Hsp70 in protecting the organism against the destructive effects of stress, and the possibility of using this protein as a marker for the development of the infarction process in the heart, the aim of this study was to conduct a comparative evaluation of expression and concentration of Hsp70, as well as the level of oxidative stress based on the concentration of TBARS and NO ions, in the muscle tissue of hearts obtained from pigs which had died with symptoms of sudden cardiac death and hearts from healthy pigs.

## Materials and Methods

The material for the study consisted of hearts obtained from 90 pigs slaughtered in a slaughterhouse and pigs which had died during transport or during rest before slaughter. The hearts were collected directly after the slaughter or death of the animal. The total number of samples in each group was 30. A macroscopic evaluation of the fresh hearts was performed according to Nachlas and Shnitka (1963). Lysates from the hearts were prepared according to Schmitt et al. (2002) with a modification by Shaila et al. (2005). A 10ml volume of pH 7.9 lysis/homogenization buffer (40 mM KCl, 3 mM $MgCl_2$, 1 mM DDT, 5% glycerol, 1 μg/ml aprotin, 1 μg/ml leupeptin, 1 mM PMSF, 0.1% Nonidet P-40) was added to each 10 g tissue sample. Fragments of the left ventricle wall and left atrium of the hearts were homogenized in an ice bath using a homogenizer (Ultra Turrax T25, D) for 15 min. To remove tissue residue, the homogenates were centrifuged at 12,000xg for 20 min. at 4°C, and the supernatant obtained was stored at -70°C until analysis.

The level of oxidative stress was determined in the lysates based on concentration of TBARS and nitrate ions. Expression and concentration of Hsp70 heat shock proteins were determined using SDS-PAGE electrophoresis, Western blotting, and an ELISA kit.

## Evaluation of oxidative stress parameters

Production of NO ions was determined as nitrite ions, using Griess reagent (Sigma-Aldrich, Germany), as the nitric oxide produced would be rapidly converted to nitrite ions (Chui et al. 2004). Each 25 µl sample was mixed with 250 µl of Griess reagent and then incubated at room temperature with no access to light for 15 min. A standard curve was prepared based on a series of dilutions of sodium nitrite solution ($NaNO_2$, Sigma-Aldrich, Ge). Absorbance was read with a spectrophotometer (BioRad, SmartSpec™ PLUS, D) at 538 nm. Results were expressed in millimoles of nitrite ions detected (mM/L). The level of nitrite ions reflected NO synthesis.

Lipid peroxidation was determined by evaluating TBARS in accordance with an earlier study (Wernicki et al. 2006). For this purpose 2.5 ml 20% TCA (trichloroacetic acid, SigmAldrich, D) in 0.6 M HCl was added to each 0.5 ml volume of the samples, and the samples were then shaken and incubated for 10 min. at room temperature. 1.5 ml 0.67% TBA (thiobarbituric acid, Sigma-Aldrich, D) in 1M NaOH was then added and the samples were incubated for 20 min. in boiling water. After cooling, 4 ml of n-butanol (POCH S.A., PL) was added and the samples were shaken for 3 min. and centrifuged for 10 min. at 4,500xg. Absorbance of the n-butanol layer was measured in a spectrophotometer (BioRad, SmartSpec™ PLUS, USA) at 532 nm. TBARS concentration in µmol/g protein was expressed as protein content (g/l). The protein content in the test lysates was determined using Bradford's reagent (Sigma-Aldrich, USA).

Analysis of Hsp70 from the heart lysates was performed using polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Separation was carried out in 10% resolving gel (pH 8.8 Tris-HCl buffer), with 4% polyacrylamide gel in pH 6.8 Tris-HCl buffer used as a stacking gel. Electrophoresis was carried out in standard Tris-glycine chamber buffer at a constant current of 50 mA at 4°C. A molecular weight standard (Page Ruler Plus Prestained Mol. Weight Standards, Fermentas, Lithuania) was used with a range of 10-250 kDa. The gels were stained with Coomassie blue (Sigma-Aldrich, D) and analysed with Quantity One software (BioRad, USA).

The protein fractions obtained were identified semi-quantitatively by Western blotting in accordance with Towbin et al. (1979) with rabbit monoclonal anti-Hsp70 antibodies (R&D, USA) as primary antibodies and horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Kom, UK) as secondary antibodies. The proteins were first transferred to a nitrocellulose membrane (0.2 µm, Millipore) at 150V for 1 hour at 4°C with a MINI V8x10 apparatus (Gibco BRL, GB). The membranes were incubated for 1.5h at room temperature in blocking buffer with 5% non-fat milk and washed twice in TweenTBS buffer (TBS buffer with 0.05% Tween 20), pH 7.5. The membranes were then incubated overnight at 4°C with primary rabbit monoclonal anti-Hsp70 antibodies, and after washing with TTBS the membranes were incubated for 60 min. at room temperature with horseradish-peroxidase-conjugated secondary goat monoclonal anti-rabbit IgG antibodies (Jackson-Immunoresearch; BioKom, UK). The membranes were stained using a Colour Development kit (BioRad, USA) and then scanned and analysed using Quantity One (BioRad, USA).

Concentrations of Hsp70 in the heart tissue were determined using an ELISA Kit assay following the producer's instructions (Cusabio Biotech Co., USA). For this purpose, 50 µl each of the lysate sample, horseradish peroxidase conjugate, and anti-Hsp70 antibodies was added to each well of the microplate. The plate was incubated at 37°C for 1 h and washed twice in buffer, and 50 µl each of substrates A and B were then added and the plate was incubated for 15 min. at 37°C without access to light. The reaction was terminated by adding 50 µl stop solution, and then read within 10 min. with an ELISA reader (BioRad, USA) at 450 nm. Hsp70 concentration was presented in ng/ml.

The results of the study were analysed statistically using Statistica 10.0. Significant differences were determined with variance analysis using the Friedman ANOVA test. Correlations between lipid peroxidation and concentration of nitrite ions and between oxidative stress and Hsp70 concentration were presented using Pearson's correlation coefficient. Differences at $p < 0.05$ were considered statistically significant.

## Results

The present study demonstrated the presence of Hsp70 in lysates obtained from the hearts of slaughtered pigs in which no macroscopic changes were noted, as well as in those obtained from pigs in which changes in the heart muscle were observed. Expression of Hsp70 was also noted in lysates from the hearts of pigs which had died with symptoms of sudden cardiac death, which was confirmed by SDS-PAGE electrophoresis (Fig. 1). Protein fractions of about 74kDa obtained in SDS-PAGE elec-

trophoresis reacted positively with mouse monoclonal anti-Hsp70 antibodies in the Western blotting (Fig. 2). The strongest reaction was observed in lysates from hearts in which no macroscopic changes were observed, while the weakest reaction was visible in the case of hearts obtained from pigs which had died with symptoms of sudden cardiac death (Fig. 2).

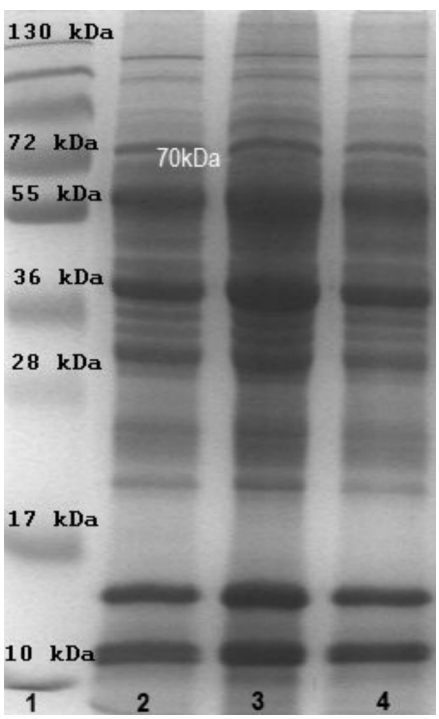


Fig. 1. SDS-PAGE electrophoresis proteins profile of the examined heart lysates obtained from pigs after slaughter or dead SDS-PAGE electrophoresis stained with coomassie blue (Sigma, Ge): Line 1 – Mol.Wt standard (10-250 kDa, Fermentas, Lithuania), 2 – protein profile of heart lysate obtained from healthy pigs; 3 – protein profile of heart lysate with post-infarction changes; 4 – protein profile of heart lysate obtained from pigs which had died with sudden heart death symptom.

Analysis of Hsp70 concentration found the highest mean values (6.63 ng/ml) in lysates from hearts in which no macroscopic infarction lesions were noted. Hsp70 concentration in hearts in which macroscopic changes were observed was slightly lower, at 4.26 ng/ml; the values obtained in comparison with the results obtained in healthy hearts were not statistically significant (Table 1). The lowest Hsp70 concentration was noted in lysates from the hearts of pigs which had died with symptoms of sudden cardiac death (macroscopically, extensive foci of necrosis were observed in the heart muscle tissue). The average Hsp70 concentration was 2.97 ng/ml and was statistically significant at $P < 0.05$ compared to the value obtained for the healthy hearts (Table 1).

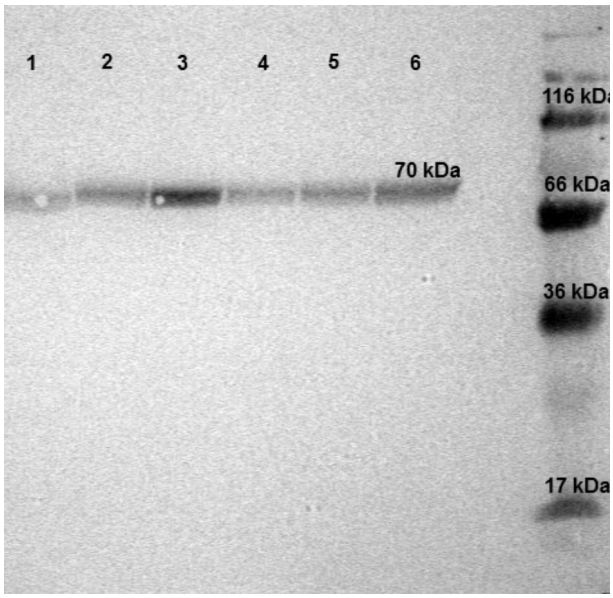


Fig. 2. Example of Western blotting reactions with mouse monoclonal anti- Hsp70 antibodies of examined heart lysates obtained from pigs after slaughter or dead. Lines 1 and 4 – reactions of heart lysates obtained from pig which had died with sudden heart death symptoms; 2 and 5 – reactions of heart lysates with post-infarction changes; 3 and 6 – reactions of heart lysates obtained from healthy pigs; Mol. Wt standard (6.5 kDa-150 kDa).

Analysis of TBARS concentration revealed the highest concentration of thiobarbituric acid reactive substances in the case of hearts obtained from pigs which had died, with values of 4.6 μM/g protein. The values obtained did not differ significantly in comparison with healthy hearts or hearts with macroscopic changes obtained from slaughtered pigs (Table 1).

Analysis of nitrite ion concentration showed the highest values (3.85 mM/l) in hearts obtained from slaughtered pigs in which macroscopic changes had been observed (Table 1). In the case of hearts of pigs which had died, the values obtained (3.23 mM/l) were not much higher than in the case of healthy animals.

The correlation observed in the study between Hsp70 concentration in the hearts of pigs which had died and those in which no necrotic lesions were noted was significant $r = 0.52$ at $p < 0.05$ (Fig. 3), while the correlation between Hsp70 concentration and nitrite ions in hearts obtained from pigs that died compared to healthy hearts was $r = 0.4$ (Table 2).

Evaluation of the correlation between Hsp70 level and TBARS concentration found the highest positive correlation ($r = 0.67$) in the case of hearts obtained from slaughtered pigs with macroscopic myocardial infarction lesions (Table 2).

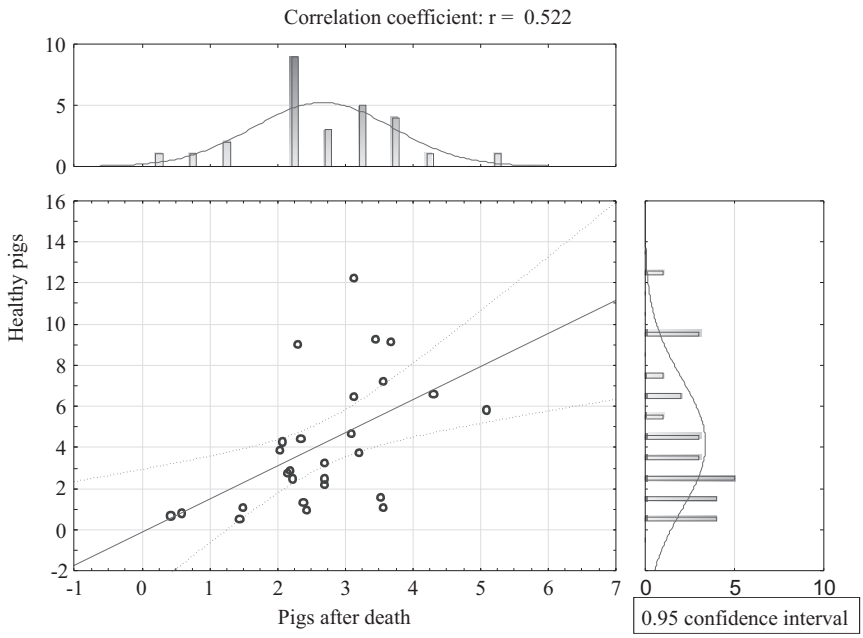


Fig. 3. Correlation between concentration of Hsp70 proteins in heart lysates obtained from healthy pigs and from pigs which had died with sudden heart death symptoms. Axis x Pigs after death; Axis y Healthy pigs; 0.95 Confidence interval

Table 1. Average concentration of TBARS, NO ions and Hsp70 proteins in heart lysates obtained from pigs after slaughter (Friedman ANOVA test).

| Estimated parameters | Healthy hearts n=30 | Hearts with post-infarction lesions n=30 | Hearts obtained from dead pigs n=30 |
|---|---|---|---|
| TBARS μM/g protein | 4.05 ± 1.2 | 3.61 ± 1.03 | 4.6 ± 1.38 |
| NO mM/l | 3.16 ± 1.39 | 3.85 ± 1.57 | 3.24 ± 1.27 |
| HSP ng/ml | 6.63 ± 2.38 | 4.26 ± 2.27 | 2.97 ± 0.83[a] |

[a] significant differences in comparison to healthy hearts ($P \leq 0.05$).

Table 2. Values of correlation coefficients between estimated parameters in hearts tissue obtained from pigs (Pearson's correlation coefficient).

| Estimated parameters | Healthy hearts n=30 | Hearts with post-infarction lesions n=30 | Hearts obtained from dead pigs n=30 |
|---|---|---|---|
| HSP70 vs. NO | | | |
| A | -0.14 | -0.15 | -0.18 |
| B | 0.1 | -0.2 | -0.19 |
| C | -0.4 | 0.1 | -0.23 |
| Hsp70 vs.TBARS | | | |
| A | 0.29 | -0.3 | 0.06 |
| B | -0.17 | 0.67* | -0.5* |
| C | 0.5* | 0.22 | 0.07 |
| TBARS vs.NO | | | |
| A | -0.35 | 0.4 | 0.46 |
| B | -0.1 | -0.18 | 0.03 |
| C | -0.3 | 0.11 | 0.34 |

A – hearts obtained from healthy pigs; B – hearts with post-infarction changes; C – hearts obtained from dead pigs; * strong correlation, $p < 0.05$.

Analysis of the correlation between concentration of thiobarbituric acid reactive substances and that of nitrite ions showed the highest correlation between the values obtained for lysates from the hearts of pigs which had died with symptoms of sudden death ($r = 0.46$). In the remaining cases the coefficients were $r \leq 0.4$ (Table 2).

## Discussion

The present study found a significant increase in oxidative stress parameters and Hsp70 level in pig hearts with macroscopic post-infarction lesions, compared to healthy hearts. The reduced expression of this protein observed in the Western blotting in the case of hearts from pigs which had died may indicate exhaustion of the organism's ability to adapt to the unfavourable change in environmental conditions, resulting in reduced production and release of Hsp70. This is also confirmed by the reduced concentration of this protein observed in the hearts of pigs which had died with symptoms of sudden cardiac death. The complex role of Hsp70 is not fully known, but according to Aggarwal et al. (2012), one of their main tasks is protection against damage to intracellular structures in organs exposed to severe stress. Other authors (Snoeckx et al. 2001, Kiang et al. 2008) have found increased expression of Hsp70 to be significantly correlated with a reduction in destructive changes in the heart muscle of rats, which may be an important element in preventing myocardial infarction in humans. It should also be emphasized that the concentration of protective Hsp70 in internal organs can undergo significant changes over time; this was demonstrated in a study by Zhang et al. (2012), in which a significant decrease was observed in the concentration of this protein in the liver and stomach of pigs slaughtered 4 hours after transport. These authors observed a slight increase in Hsp70 levels in the heart tissues after 1 h of transport, a slight reduction after 2 h of transport, and then again an increase after 4 h of transport. The authors suggest that the changes in Hsp70 protein expression could result from differences in consumption of the Hsp70 protein during or after its protective functions during transportation.

In another study (Bao et al. 2008), a significant increase in Hsp70 concentration was observed in the heart muscle of pigs following transport; it decreased substantially 6 hours after transport, which the authors suggest may be due to the reduced need for them when the animals no longer had to maintain a standing position in a moving vehicle. According to Hao et al. (2010), the reason for changes in Hsp70 expression and levels in heart tissues is still unclear and in need of further investigation.

The changes in lipid peroxidation final products and nitrite ions shown in the study also indicate disruptions in antioxidative/oxidative status induced by health disorders in the transported pigs.

The high correlation ($r > 0.5$) observed in the study between TBARS and Hsp70 concentration in the hearts of healthy pigs and those that died indicates a significant relationship between increased lipid peroxidation rate and Hsp70 production. The somewhat weaker correlation ($r = 0.46$) between the concentration of nitrite ions and Hsp70 level in the case of healthy hearts and those obtained from pigs which had died may indicate that possibilities for using this parameter as a marker of myocardial infarction are limited.

Evaluation of oxidative stress parameters and Hsp70 concentration in pigs, both intravital and post-mortem, is the subject of experiments conducted in many research facilities. This is because pigs are physiologically and anatomically very similar to humans (metabolism, cardiovascular disease entities, and the possibility of xenotransplantation), so they are an excellent model in alternative research evaluating the predisposition for many disease entities, including myocardial infarction (Neri et al. 1995).

The results obtained confirm the significant effect of transport stress and slaughter on the level of oxidative parameters and Hsp70 concentration in the heart muscle of pigs. The study showed that the level of this protein was substantially reduced in hearts obtained from pigs which had died with symptoms of sudden cardiac death, which may indicate that the protein has a significant role in preventing death in pigs intended for slaughter. It may also be suggested that alongside troponin, myoglobin, creatine kinase, and other biochemical markers (Kemp et al. 2004, Mcdonnel et al. 2009), Hsp70 concentration can be used as one of the intravital diagnostic indicators of the infarction process and the stress reaction in people and animals. It should be emphasized that unlike troponin, myoglobin, or creatine kinase, whose maximally elevated concentration is maintained for a period of 12-24 hours after the disease process begins, changes in Hsp70 level are observed for a much shorter period of about 6-8 hours. Due to the substantial variation observed in Hsp70 concentration within an animal species, it is currently difficult to determine the range of physiological norms for the parameter, the level of which changes during a period of up to a few hours after the appearance of the stress factor (Yu et al. 2007, Bao et al. 2008, Zhang et al. 2012).

## Conclusion

To sum up, it can be concluded that the significant reduction in Hsp70 concentration noted in the hearts of pigs which had died with symptoms of sudden cardiac death compared with the control may indicate exhaustion of adaptive mechanisms in the organism, resulting in a lack of protection of internal organs against the destructive effects of stress, which is manifested in the occurrence of stress cardiomyopathy. This may also indicate that sufficient induction of Hsp70 may protect the myocardium from ischemia. Further research should be conducted to determine what level of this protein indicates a risk of sudden cardiac death, and whether it can be determined while the animals are alive.

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