

## Difference in the expression of CIRBP, RBM3 and HSP70 in the myocardium and cerebellum after death by hypothermia and carbon monoxide poisoning

Satomu Morita<sup>1</sup>, Satoshi Furukawa<sup>1,\*</sup>, Lisa Wingenfeld<sup>2</sup>, Wakoto Matsuda<sup>1</sup>, Katsuji Nishi<sup>1</sup>

**Abstract:** We studied the expression of hypoxia-related antigens (e.g., cold-inducible antigens and apoptotic antigens) in the myocardium and the cerebellum that were obtained from individuals after death by carbon monoxide or hypothermia. The immunohistochemistry results revealed that expression of cold-inducible RNA binding protein (CIRBP) and RNA-binding protein 3 (RBM3) may be associated with hypothermic and the hypoxic conditions. The expression of CIRBP and RBM3 in the myocardium was different from their expression in the cerebellum, especially in the Purkinje cells. The results indicate that agonal duration influences antigen expression. In the hypothermic condition, the myocardium uses more ATP since the force of the excitation-contraction coupling of the myocardium increases by more than 400% when the experimental temperature is reduced from 35°C to 25°C. The results obtained in this study indicate that physicians should pay attention to the myocardium when cooling the patient's body to protect the brain.

**Key Words:** carbon monoxide death, cerebellum, CIRBP, hypothermic death, myocardium, RBM3.

Hypothermia commonly results from an injury in a cold environment, immersion in cold water, or prolonged exposure to low temperatures without adequate protective clothing and equipment [1]. A diagnosis of environmentally induced hypothermia is difficult to affirm at forensic autopsy without a thorough review of the circumstances.

Hypothermia affects the cardiovascular, hematological, neurological, respiratory, renal, metabolic, and gastrointestinal systems [2]. Body temperature affects the affinity between oxygen and hemoglobin. Decreasing the dissociation of oxygen from hemoglobin is expected to occur easily in the hypothermic condition. Carbon monoxide (CO) poisoning causes myocardial toxicity and life-threatening arrhythmias [3]. The carboxyhemoglobin (COHb) level is correlated with the prolongation of QT intervals and the release of cardiac

enzymes [3].

The binding of carbon monoxide to cytochrome oxidase also interferes with aerobic metabolism and with efficient adenosine triphosphate (ATP) synthesis [3]. These conditions seem to be similar to conditions that are present in hypothermic death since the lack of oxygen or a decrease in the release of oxygen results in decreased ATP production in a tissue [4, 5]. The death mechanisms of hypothermia and carbon monoxide poisoning are similar [6]. We previously indicated that cardiac hypoxia was caused by a disturbance in the release of oxygen from the hemoglobin molecule in hypothermia or caused by a lack of oxygen because of the high affinity of carbon monoxide for hemoglobin in CO poisoning.

These results led us to study whether immunohistochemical differences exist in the myocardium and in the cerebellum in both conditions. In

---

1) Department of Legal Medicine, Shiga University of Medical Science, Japan

\* Corresponding author: MD, Department of Legal Medicine, Shiga University of Medical Science, Setatsukinowa, Otsu City, Shiga 520-2192, Japan, Tel/ Fax: +81-77-548-2200, E-mail: 31041220@belle.shiga-med.ac.jp

2) Institute of Legal Medicine, Munich University, Germany

this study, we present some discrepant results obtained from several autopsy cases of individuals who died by accidental hypothermia or by suicidal carbon monoxide poisoning.

## MATERIALS AND METHODS

We selected several individuals who died by hypothermia or by carbon monoxide poisoning. Table 1 lists their characteristics.

The remaining ratio of granular cells in the cerebellum of each individual was determined, based on our previous report [7]. The typical death case

**Table 1.** Summary of individuals examined and staining results

No.	Age	Sex	CO %	Cause of death	Postmortem Interval	Postmortem Interval %
1	39	M	93.7	CO; charcol	25 hours	29.7
2	41	F	82.8	CO; charcol	24 hours	27.7
3	46	M	78.4	CO; charcol	4 days	29.7
4	40	M	80	CO; charcol	7 days	32.7
5	38	M	78.5	CO; charcol	7 days	21.4
6	26	M	87.1	CO; charcol	6 days	24.3
7	25	F	81.6	CO; charcol	6 days	26.8
8	55	M	84.1	CO; charcol	3 days	22.8
9	57	F	79.4	CO; charcol	3 days	33.7
10	26	F	0	hypothermia	20 hours	49.6
11	60	M	0	hypothermia	48 hours	39.2
12	63	F	0	hypothermia	48 hours	48.9
13	89	M	0	hypothermia	4 days	49.5
14	85	F	0	hypothermia	36 hours	52.7

CO; saturation of COHb, CO; charcol; suicidal CO poisoning, Remaining GC; Remaining granular cells in the cerebellum.

**Table 2.** Characteristics of antibodies used in this study

Antibody	Maker	Clone	Species	AG-retrieval	Incubation	AB-dilution
CIRBP	Protein Tech	10209-2-AP	R	autoclave	overnight	1:400
RBM3	Protein Tech	14363-1-AP	R	autoclave	overnight	1:400
HSP70	santa cruz	polyclonal	G	autoclave	overnight	1:400
HIF-1 $\alpha$	Novus	NB100-479	R	autoclave	overnight	1:400
VEGF	Milipore	JH121	M	autoclave	overnight	1:400
eNOS	Gene Tex	polyclonal	R	autoclave	overnight	pre-diluted
AIF- $\alpha$	LSBio	aa-593-606	R	autoclave	overnight	1:400
P53	santa cruz	FL-393	G	autoclave	overnight	1:400
cFOS	Gene Tex	polyclonal:	R	autoclave	overnight	1:800
Ngb	SIGMA-ALDRICH	polyclonal:	R	autoclave	overnight	1:400
Wnt	Novus	6F2	M	autoclave	overnight	1:400
SIRT1	Novus	E104:	R	autoclave	overnight	1:400
CCC9	Leica	10A6	M	autoclave	overnight	1:400
Carbindin	santa cruz	sc-58699	M	autoclave	overnight	1:400

characteristics of the individuals were as follows:

### *Hypothermic Death (Autopsy Number 10)*

A 26-year-old female was found dead on a road during a winter morning. The autopsy revealed that postmortem lividity on her back was clear red, and the blood was reddish on the left side of her heart and in both lungs. Wischniewski ulcers were detected in the stomach mucosa. Her postmortem interval was estimated at 20 hours. The ratio of remaining granular cells in the cerebellum was calculated as 49.6%.

### *Carbon Monoxide Poisoning Death (Autopsy Number 2)*

A 41-year-old female was found dead in her lover's bedroom. A police investigation revealed a small portable stove that contained burned up charcoal briquettes.

The postmortem lividity on her back was clear scarlet. The autopsy revealed that the woman died of carbon monoxide poisoning. The estimated postmortem interval was 24 hours. The ratio of remaining granular cells in the cerebellum was calculated as 27.7%.

At each autopsy, we usually collected four sections of the heart and one section of cerebellum containing the dentate nucleus. The tissue sections were fixed in formalin and prepared in paraffin blocks within three days after the autopsies. Immunohistochemical examination of these tissues was performed by using the ABC technique (Nichirei, Tokyo, Japan) and using antibodies against cold-inducible RNA binding protein (CIRP), RNA-binding protein 3 (RBM3), heat shock protein 70 (HSP70), hypoxia-inducible factor 1 (HIF1), vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (e-NOS), allograft inflammatory factor 1 (AIF1), cFos, neuroglobin (Ngb), Wnt, cation-chloride cotransporter 9 (CCC9), carbindin, and sirtuin 1 (SIRT1).

Table 2 lists the characterization of the antibodies. Each staining procedure was performed in accordance with the manufacturer's protocol. The characteristics of the antibodies are described in other reports [5].

## RESULTS

### *The Myocardium*

**Hypothermic Death.** Antibodies against CIRP, RBM3 and SIRT1 showed good reactivity with the nucleus of cells of the myocardium (Fig. 1). These antibodies also showed weak reactivity with the cytoplasm of myocardial cells that were located around the contraction band necrosis. The antibodies showed no reactivity in the area of contraction band necrosis in the myocardium. Staining intensity of these antibodies was, in decreasing order, RBM3, CIRP, and SIRT1.

Anti-HSP70 showed granulated reactivity with the cytoplasm of cells located around the contraction bands. This antibody showed no reactivity with the nuclei of cells in the myocardium. Antibodies such as AIF1, protein 53 (p-53), HIF-1, VEGFA, and e-NOS showed moderate reactivity in the cytoplasm of cardiac cells containing contraction band necrosis and cells existing around the contraction band necrosis. Anti-Ngb, anti-Wnt and anti-CCC9 showed no reactivity in the cytoplasm and nuclei of myocardial cells. The reactivity of antibodies against RBM3, e-NOS, and CCC9 was detected in the epithelial cells of blood vessels.

**Carbon Monoxide Poisoning Death.** Anti-CIRBP and anti-RBM3 showed moderate reactivity with the nucleus of cardiac cells in carbon monoxide death, compared to hypothermic death.

The number of positive cells were smaller in carbon monoxide poisoning death than in hypothermic death (Fig. 1). These antibodies showed moderate reactivity with the cytoplasm of cells located around the contraction band necrosis. Anti-HSP70 showed granulated reactivity with the cytoplasm of the cells

located around the contraction band. However, this antibody showed no reactivity with the nuclei of the myocardial cells. Anti-HIF1, anti-VEGF, anti-AIF, and anti-p53 antibodies showed weak or moderate reactivity only with the cytoplasm of cardiac cells located around the contraction band necrosis.

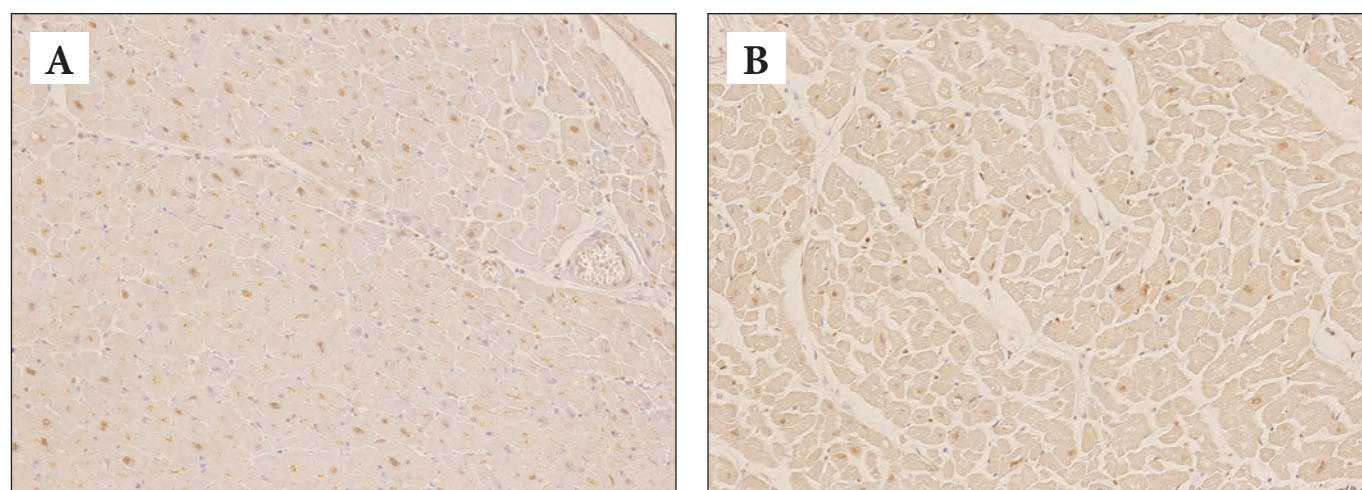
Anti-e-NOS showed intense and granulated reactivity with the cytoplasm of the cardiac cells located around the contraction band necrosis, but no reactivity with the nucleus. No reactivity of anti-Ngb, anti-Wnt, and anti-CCC9 was observed in the myocardium, although blood vessel endothelial cells showed good reactivity with anti-CCC9. Anti-SIRT1 showed weak to moderate reactivity with the nucleus in a few cardiac cells that were located far from the contraction bands. The reactivity of anti-SIRT1 was also clearly weak, compared to the reactivity of anti-CIRBP and anti-RBM3.

The frequency of the reactivity of SIRT 1 with the nuclei of cells in the myocardium was lower in the carbon monoxide death cases than the reactivity in the hypothermic death cases. Anti-SIRT 1 also showed granulated reactivity in the cytoplasm of the cells that were located far from the contraction bands.

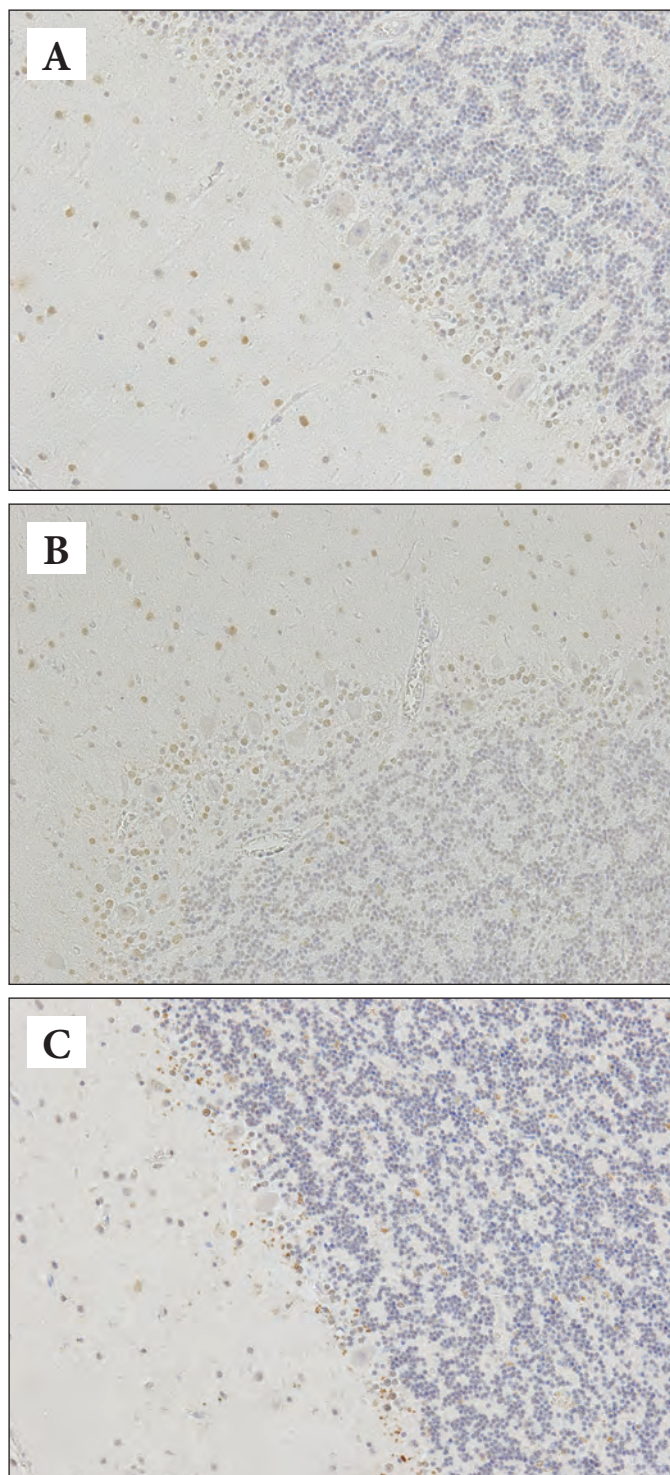
### *The cerebellum*

Anti-carbindin, which is specific for Purkinje cells, clearly stained the cytoplasm of cells in both types of deaths.

**Hypothermic Death.** Anti-CIRBP, anti-RBM3, and anti-HSP70 showed good reactivity with the cells of the molecular cell layer and the Purkinje cell layer, and they showed weak reactivity with cells in the granular cell layer. However, these antibodies had a feeble reactivity with the Purkinje cells (Fig. 2). Anti-HIF1 and anti-e-NOS showed moderate or weak reactivity with the Purkinje cells, whereas anti-VEGF showed no reactivity with the Purkinje cells. These antibodies showed weak reactivity with cells in the molecular layer. Anti-Ngb and anti-AIF1 showed weak reactivity with the cytoplasm

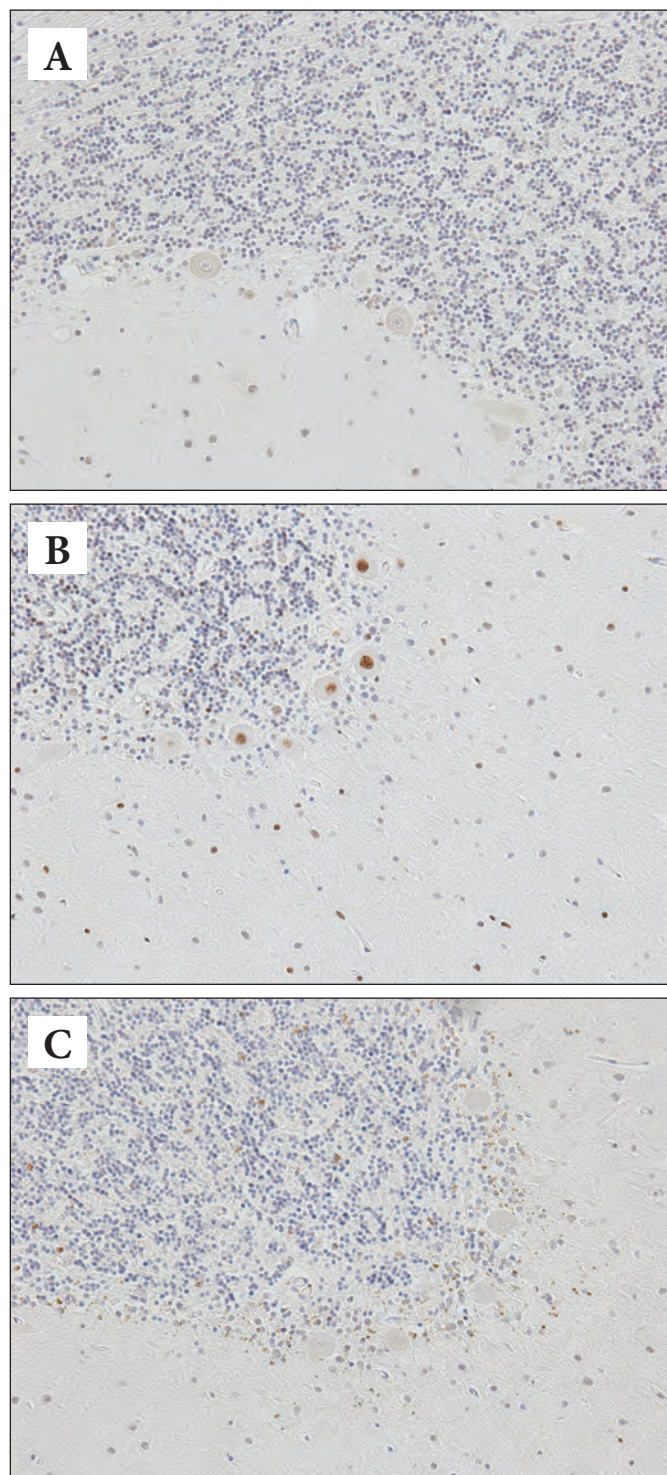


**Figure 1.** Anti-RBM3 antibody staining of the myocardium obtained from individuals who died by (A) hypothermia or (B) carbon monoxide poisoning (magnification, 400×). The number of stained nuclei is greater in hypothermic death than in carbon monoxide poisoning death. In hypothermic death, the myocardial cells are gathered by each other.



**Figure 2.** The cerebellum of individuals who died by hypothermia. The cerebellum is stained by (A) anti-CIRBP, (B) anti-RBM3, and (C) anti-HSP70 (magnification, 400×). The antibodies stained the cells in the molecular layer and did not stain the Purkinje cells. Anti-HSP70 stained many cells in the Purkinje cell layer.

of the Purkinje cells and anti-p-53 showed moderate reactivity in the cells of the granular cell layer. Anti-Wnt, anti-c-Fos, anti-SIRT 1, and anti-CCC9 showed no reactivity in the Purkinje cells, and anti-SIRT1 showed weak reactivity with the cells of the molecular layer.



**Figure 3.** Staining of the cerebellum after carbon monoxide poisoning by (A) anti-CIRBP, (B) anti-RBM3, and (C) anti-HSP70 (magnification, 400×). Anti-CIRBP stained the cytoplasm of the Purkinje cells. Anti-RBM3 stained the nuclei of the Purkinje cells. Anti-HSP70 did not stain the Purkinje cells. HSP70 stained the cells in the Purkinje cell layer. Some cells in the molecular layer reacted with these antibodies.

The reactivity was generally weak in individuals who had died of hypothermia, compared to the reactivity in the cerebellum obtained from individuals who had died of carbon monoxide poisoning.

*Carbon Monoxide Poisoning Death*

The nuclei of the Purkinje cells were clearly stained by anti-RBM3 (Fig. 3). The cytoplasm of the Purkinje cells showed no reactivity with this antibody. Granular cells and some cells in the molecular layer showed weak reactivity with antibodies against CIRBP, RBM3, and HSP70. Anti-CIRBP and anti-HSP70 showed feeble or no reactivity with the Purkinje cells. Anti-HSP70 stained some cells in the Purkinje cell layer (Fig. 3). Anti-HIF1 weakly stained the cytoplasm of the Purkinje cells, and anti-VEGF and anti-e-NOS slightly stained the cytoplasm. These antibodies stained some cells in the molecular layer. Anti-e-NOS stained some cells in the granular cell layer. Anti-Ngb and anti-SIRT1 showed weak or moderate reactivity with the cytoplasm of the Purkinje cells, although anti-Wnt, anti-AIF-1, anti-c-Fos, anti-p-53, and anti-CCC9 showed no reactivity with the Purkinje cells. Anti-Ngb and anti-SIRT1 showed weak reactivity with some cells in the molecular layer.

**DISCUSSION**

The expression of antigens such as CIRBP, RBM3, and HSP70 in the myocardium was slightly weak in the carbon monoxide poisoning death cases, compared to their expression in the hypothermic death cases. However, the expression by anti-RBM3 in the Purkinje cells was intense and clear in the nuclei of the Purkinje cells obtained from individuals who had died by carbon monoxide poisoning.

The staining intensities by the same antibodies differed between the myocardium and the cerebellum. Scientists originally discovered CIRBP and RBM3 as cold-inducible RNA binding proteins [8, 9]. However, CIRBP and RBM3 are reportedly adaptatively expressed in response to hypoxia by a mechanism that involves neither HIF1 nor the mitochondria [10]. We also showed that CIRBP and RBM3 were detected in the hypoxic state; this confirms the present proposal [11]. The difference in the expression of these antigens in the myocardium after hypothermic death or carbon monoxide death may be caused by the agonal duration of the individuals.

The agonal duration in hypothermic death presumes several hours and the duration of carbon monoxide death is estimated within one hour. The difference of self-inflicted hypoxia and agonal duration may cause a different progression in the signal pathway and cascade process in the myocardium. The difference in the myocardium may be easily understood. However, the expression of these antigens in the cerebellum differed from their expression in the myocardium. The nuclei in Purkinje cells were clearly stained by anti-RBM3 in carbon monoxide poisoning death. On the other hand, the reactivity of anti-CIRBP and anti-RBM3 were weak

in hypothermic death, although anti-HSP70 showed relatively similar reactivity with cells in the Purkinje cell layer and molecular layer.

The reactivity of anti-HIF-1, anti-VEGF, and anti-e-NOS with the cells of the cerebellum was also discrepant. The discrepancy between the myocardium and the cerebellum was not able to be resolved by only the difference in agonal duration between the two types of death conditions. The neuroprotective effects of cooling the body have largely been attributed to the finding that lowered temperatures decreases the metabolic rate and reduces blood flow in the brain [12]. Hypothermia also affects other processes associated with ischemia such as the induction of immediate early gene expression and the cellular stress response [13]. The process by which excitation of the myocyte leads to cardiac contraction is usually known as excitation-contraction (EC) coupling [14].

For the last several decades, investigations of the mechanisms involved in EC coupling in the mammalian heart have been conducted in isolated cardiac myocytes at room temperature. Since mammalian core temperatures are generally in the range of 35°C to 39°C, studies of mammalian cells conducted at room temperature (i.e., 20°C–24°C) have explored cardiac function under hypothermic conditions. It has long been established that hypothermia is a potent positively inotropic stimulus that causes a marked increase in the magnitude of cardiac contraction [15].

Shattock and Bers described that when the temperature is reduced from 37°C to 25°C, the force produced by rabbit ventricular muscle is increased by more than 400% [16]. The amplitude of contractions recorded from the ventricular myocytes of rabbit, cat, and ferret increases markedly when the temperature is reduced from 35°C to 25°C [17]. These previously reported observations indicate that temperature can affect mechanisms that are important in cardiac EC coupling and indicate that cooling increases the action potential duration in cardiac myocytes and ventricular muscle preparations from rabbit hearts [16]. The increased excitation of the myocardium in hypothermia reportedly decreases the ATP concentration in cells. The decrease in ATP may stimulate the signal pathway processes in the myocardium. On the other hand, the hypothermia disturbs the metabolic rate in cerebellar cells, but with little consumption of ATP. The Purkinje cells in the hypothermic condition showed weak reactivity with the hypoxia-related antibodies. The results obtained in the present study may be confirmed by reports described in the past several decades in the biophysical field. The results also indicate that physicians should pay attention to the myocardium when cooling a patient's body to protect the brain.

## References

1. Weinberg AD. Hypothermia. *Ann Emerg Med* 1993; 22: 370-737.
2. Mallet M L., Review: Pathophysiology of accidental hypothermia. *QJ Med*; 2002; 95:775-785.
3. Yelken B, Tanriverdi B, Cetinbas, Memis D, Suet N, The assessment of QT intervals in acute carbon monoxide poisoning, *Anadolu Kardiyol Derg* 2009; 9: 397-400.
4. Furukawa S, Wingensfeld L, Sakaguchi I, Nakagawa T, Takaya A, Morita S, Yamasaki S, Nishi K, Histopathological characteristics of human cardiac tissues in accidental hypothermia using conventional staining techniques. *Rom J Legal Med*, 2012; 20: 241-246.
5. Furukawa S, Wingensfeld L, Sakaguchi I, Nakagawa T, Takaya A, Morita S, Yamasaki S, Nishi K, Histopathological characteristics of human cardiac tissues in accidental hypothermia using immunohistochemistry: Immunohistochemical detections of cold, hypoxia and apoptotic related antigens are practicable in the myocardium from hypothermic death even in postmortem examination, *Anil Aggrawal Internet Journal of Forensic Medicine and Toxicology*, 2013;
6. Morita S, Furukawa S, Wingensfeld L, Nishi K, Pathological findings of myocardium in carbon monoxide poisoning are basically similar to those in hypothermic deaths. *Anil Aggrawal Internet Journal of Forensic Medicine and Toxicology*, 2013;
7. Morita S, Furukawa S, Takaya A, Sakaguchi I, Okunaga H, Nakagawa T, Wingensfeld L, Nishi K, Useability of histological assessment of cerebellar granular cell layer regardless of postmortem interval, *J Forensic Res*, 4:180. 2013.
8. Nishiyama H, Higashitsuji H, Yokoi H, Itoh K, Danno S, Matsuda T, Fujita J, Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene, *Gene* 1998; 204: 115-120, 1998.
9. Derry J M, Kerns J A, Francke U, RBM3, a novel human gene in Xp11.23 with a putative RNA-binding domain, *Hum Mol Genet*, 1995; 4: 2307-2311.
10. Wellmann S, Buhner C, Moderegger E, Zelmer A, Kirschner R, Koehne P, Fujita J, Seeger K., Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J Cell Sci* , 2004; 117: 1785–1794.
11. Furukawa S, Sakaguchi I, Morita S, Nakagawa T, Takaya A, Wingensfeld L, Nishi K, Suicidal ligature strangulation without an auxiliary mechanism. Reports of two cases with a cotton rope or a T-shirt and staining results of the brains using anti HSP70, CIRBP, RBM3, HIF-1-alpha, SIRT1 and p 53 antibodies, *Rom J Legal Med*, 2013, 21, 9-14.
12. Erecina M, Thorensen M, Silver I A, Effects of hypothermia on energy metabolism in mammalian central nervous system, *J Cere Blood Flow Metab*. 2003; 23: 513-530.
13. Kamme F, Campbell K, Wieloch T, Biphasic expression of the fos and jun families to transcription factors following transient forebrain ischaemia in the rat. Effect of hypothermia. *Eur J Neurosci*. 1995; 7: 2007-2016.
14. Bers DM. Cardiac excitation-contraction coupling. *Nature* 415: 198–205, 2002.
15. Blinks JR, Koch-Weser J. Physical factors in the analysis of the actions of drugs on myocardial contractility. *Pharmacol Rev*, 1963; 15: 531–599.
16. Shattock MJ, Bers DM. Inotropic response to hypothermia and the temperature-dependence of ryanodine action in isolated rabbit and rat ventricular muscle: implications for excitation-contraction coupling. *Circ Res*, 1987; 67: 761–771.
17. Puglisi JL, Bassani RA, Bassani JW, Amin JN, Bers DM. Temperature and relative contributions of Ca transport systems in cardiac myocyte relaxation. *Am J Physiol Heart Circ Physiol*, 1996; 270: 1772–1778.