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Expression of antigens related to hypoxia, stress, and apoptosis in the myocardium after fatal carbon monoxide exposure

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Abstract: Although it has been described and generally accepted that the myocardium does not show any ischemic types of changes or other lesions in fatal carbon monoxide poisoning victims, we previously observed detailed pathological findings with careful staining using HE and/or Azan. We have used immnohistochemistry on the myocardium from twenty victims of both carbon monoxide poisoning and house fire. We especially selected antibodies which were previously recognized as cold stress antigens. And we also used antibodies related to stress, apoptosis, and vascular genesis. The nuclei showed good reactivity with CIRBP, RBM3, and SIRT1 antibodies. Although CIRBP and RBM3 were previously recognized as cold stress antigens, this study indicated that these antigens were also transcribed in hypoxia. Antigens related to stress, apoptosis, and vascular genesis were also detected in the myocardium from carbon monoxide poisoning victims. The results obtained in the present study suggest that antigens, such as CIRBP, RBM3 and SIRT1, may be useful temporal markers of global hypoxia or ischemia in myocardium. This study also found that the cells once exposed to stress and hypoxia, develop and activate signal pathways to prevent harmful consequences, and the expressed antigens may be retained after cell death and during the postmortem interval.
Key Words: Carbon monoxide poisoning, Hypoxic cardiac cells, CIRBP, RBM3, SIRT1.

It has been reported and generally accepted that the myocardium does not show any ischemic types of changes or other lesions in fatal carbon monoxide poisoning cases [1]. However, some reports indicated the existence of pathological changes in the myocardium from carbon monoxide poisoning cases [2, 3]. In fatal carbon monoxide poisoning, the saturation of hemoglobin with carbon monoxide exceeds 70% and the lack of oxygen can be expected to cause damage in the myocardium. In forensics, there are two situations in which the oxygen concentration is slowly decreased and is finally depleted. The first is hypothermic death and second is carbon monoxide poisoning. In our previous study, we examined the expression of hypoxia, stress, and apoptosis related antigens in the myocardium obtained from individuals who died from hypothermia [4], and our findings led us to further investigate the expression of these antigens in the myocardium of fatal carbon monoxide poisoning cases.

MATERIALS AND METHODS

We selected 9 individuals who died due to carbon monoxide poisoning. In one case, 5 unrelated victims died in a car, and in the other cases, 5 persons died in separate cars. We also studied victims of carbon monoxide from house fires. Four victims died in their own houses due to burning charcoal briquettes. We additionally selected 5 persons with/or without COHb, who died in house fires. All died without resuscitation attempts of any

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type. Additionally we added several hypothermic death cases, one asphyxia case in which a man died due to a fall into an industrial stirrer filed with nitrogen gas, and a cardiac infarction case with tamponade as comparison cases. The age, gender, cause of death, saturation level of COHb, and death scene situation of each victim are described in Table 1. We routinely examined four parts of the heart: the horizontal sections from both the right and left ventricle and septum and the longitudinal sections of the interventricular septum, including the conduction system. All sections were stained with HE and/or Azan.

The levels of COHb in the blood from each individual were determined by a visible spectrophotometry method [5]. We considered the presumed agonal duration of the individuals examined in this study. The concentration of carbon monoxide would be expected to increase gradually in the suicidal cases where one or two pieces of charcoal briquette in a small portable stove for cooking were used for generation of carbon monoxide whereas in the victims of a house fire the level of carbon monoxide would rapidly increase. The agonal duration was presumed to be longer in the suicidal cases. To evaluate the agonal duration, we examined the degree of degeneration in the granular cell layer of the cerebellum obtained from each individual. We evaluated the granular cells in the cerebellum in a similar manner to our previous study [6] using a microscope and a Photoshop application. Photographs at the same magnification were made of the granular layer after staining with hematoxylin, which stains the viable nuclei blue, and the area of blue staining, was measured. The amount of remaining granular cells was indicated as a percentage of the total number of pixels. The numerical value was the mean value of five different areas. The age, gender, cause of death, saturation level of COHb, remaining granular cells, and death scene situation are described in Table 1.

We stained the cardiac tissues after preparing 4 μm paraffin sections using standard immnunohistochemical techniques. We utilized antibodies against Cold-inducing RNA-binding protein (CIRBP) [7], RNA binding motif 3 (RBM3)[8), Sirtuin 1 (SIRT1) [9], and heat shock protein 70 (HSP70) [10]. We also used antibodies against hypoxia-inducing factor 1 alpha (HIF1α) [11], Vascular endothelial growth factor (VEGF) [12], endothelial cell nitric oxide synthase (eNOS) [13], apoptotic-inducing factor 1 (AIF-1) [14], and Complement components C4 and C9 [15, 16]. The characteristics of the antibodies used are listed in Table 2. The staining procedure (LSAB method) was similar to that of our previous study [6].

RESULTS

Conventional staining with HE and Azan revealed the number of red - or orange-colored cardiac cells (colored cell), frequency of colliquative myocytolysis cells, and contraction bands in the myocardium as shown in Fig. 1.

In the present study, the nuclei in the cardiac cells were stained by anti CIRBP, RBM3, and SIRT1

Table 1. Summary of individuals examined and staining results

No.	Age	Sex	CO(%)	Cause of death	PMI	GC(%)
1	39	M	93.7	CO; charcol	25H	29.7
2	41	F	82.8	CO; charcol	25H	27.7
3	46	M	78.4	CO; charcol	4D	29.7
4	40	M	80	CO; charcol	7D	32.7
5	38	M	78.5	CO; charcol	7D	21.4
6	26	M	87.1	CO; charcol	6D	24.3
7	25	F	81.6	CO; charcol	6D	26.8
8	55	M	84.1	CO; charcol	3D	22.8
9	57	F	79.4	CO; charcol	3D	33.7
10	43	F	79.5	house fire	30H	45.7
11	13	F	89.5	house fire	30H	44.2
12	16	F	83.5	house fire	30H	51.7
13	62	F	0	fire; suicide	30H	46
14	59	M	0	fire; suicide	24H	65
15	26	F	0	hypothermia	30H	56.3
16	89	M	0	hypothermia	4D	-
17	**85**	**F**	**0**	**hypothermia**	**36H**	**-**
18	**32**	**M**	**0**	**asphyxia**	**15H**	**-**
19	**51**	**M**	**0**	**cardiac tamponade**	**36H**	**25.6**
20	**82**	**M**	**0**	**cardiac tamponade**	**36H**	**27.7**

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

Table 2. Characteristics of the antibodies used

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	1:200
SIRT1	Novus	E104:	R	autoclave	overnight	1:400
HSP70	Santa Cruz	polyclonal:	G	autoclave	overnight	1:400
eNOS	Gene Tex	polyclonal:	R	autoclave	overnight	pre-diluted
HIF-1α	NOVUS	NB100-479	R	autoclave	overnight	1:400
AIF-α	LSBio	aa-593-606	R	autoclave	overnight	1:200
P53	Santa Cruz	FL-393 :	G	autoclave	overnight	1:400
VEGF	Milipore	JH121	M	autoclave	overnight	1:400
CCC9	Leica	10A6	M	autoclave	overnight	1:400
C4d	ARP		R	autoclave	overnight	1:400

AG;Antigen, AB;Antibody

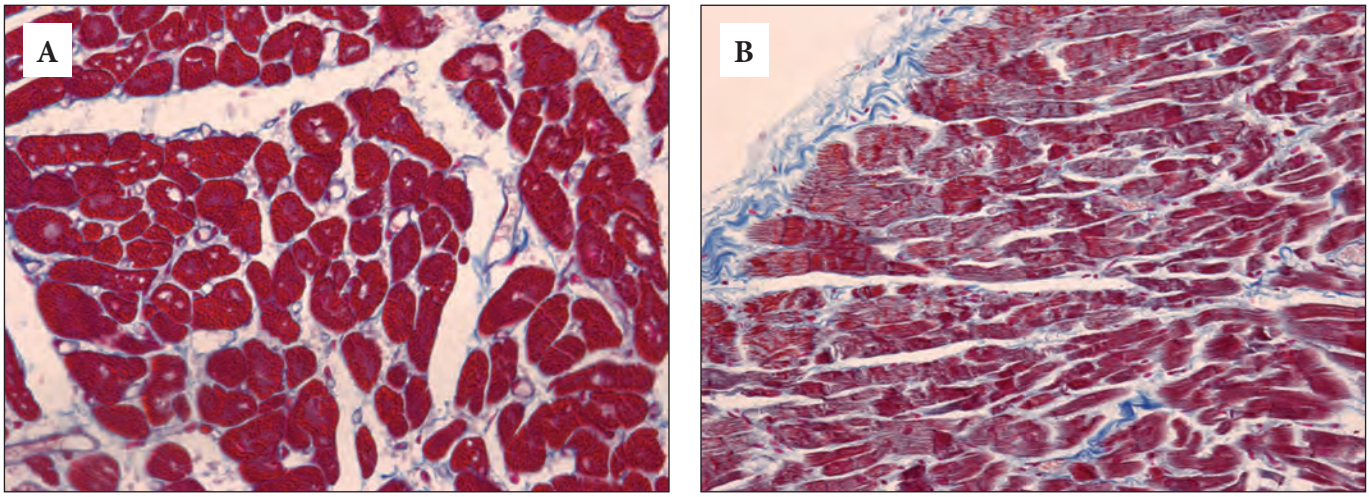

Figure 1. Hypoxic changes observed in the myocardium using Azan stain. Azan stain showed orange color myocardial cells in the left ventricle (A) and contraction bands and colliquative myocytolysis (B) were also recognized in the right ventricle obtained from the individual who died carbon monoxide poisoning using burning charcoal briquette.(No.2 individual in Table1).

antibodies, although the intensity and frequency of reactivity were different. These antibodies also showed reactivity of different intensities in the cytoplasm.

Anti CIRBP and RBM3 moderately reacted with the nuclei in the cardiac cells without colliquative myocytolysis and/or hypereosinophilic changes from all individuals who died due to suicidal carbon-monoxide poisoning (Fig. 2), and weak reactivity was observed in the nuclei of cardiac cells from individuals who either died in house fires, due to cardiac tamponade, and/or asphyxia. Very weak reactivity was observed in the nuclei from individuals who died due to self-immolation and had no COHb in their blood. Anti RBM3 antibody showed clearer and more intense reactivity in the nuclei than that observed with anti CIRBP antibody (Fig. 2). Anti CIRBP and RBM3 antibodies also stained the cytoplasm of the colliquative myocytolysis and/or hypereosinophilc cells.

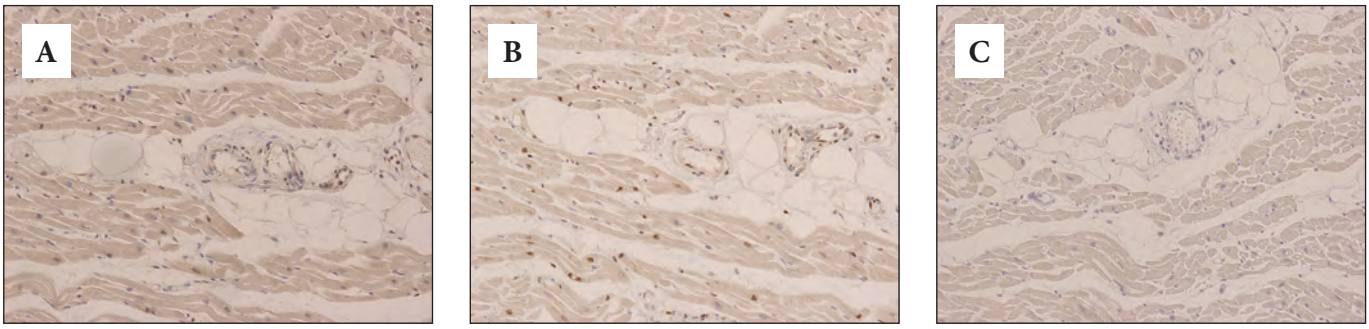

Figure 2. Staining results by anti CIRBP(A), RBM3(B) and SIRT1(C). Anti CIRBP and RBM3 stained the nucleus, but anti SIRT 1 showed weak reactivity with the nucleus of the myocardial cells obtained from carbon monoxide poisoning individual. (No.1 individual in Table 1).

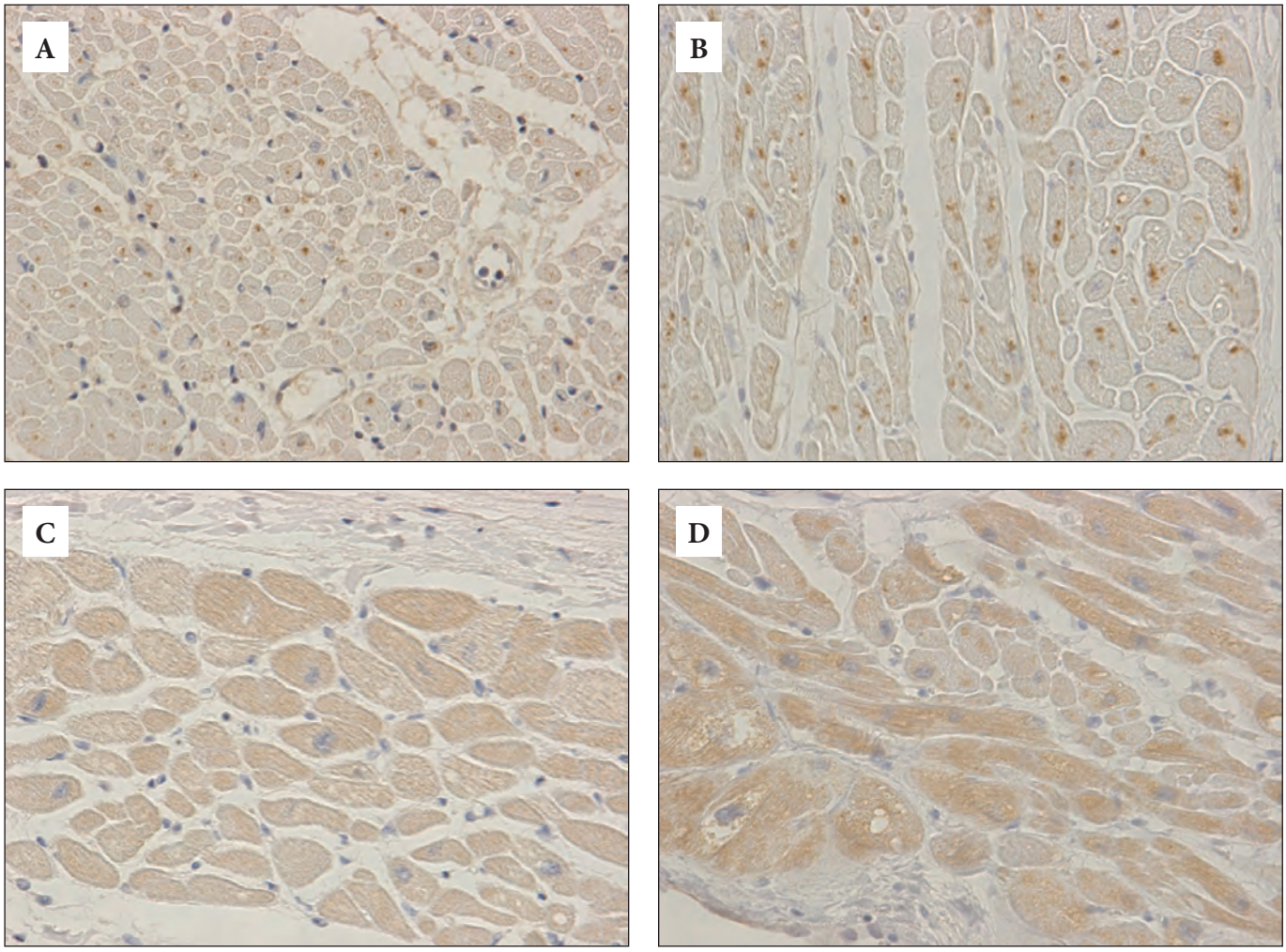

Figure 3. Staining results of the myocardial cells obtained from carbon monoxide poisoning individual by anti HSP70, eNOS, HIF1α and AIF. Anti HPS70 (A) and eNOS (B) showed granulated reactivity in the cytoplasm. Anti HIF (C) and AIF (D) showed weak but diffuse reactivity with cytoplasm. (No.1 individual in Table1).

Anti SIRT1 antibody showed weak to moderate reactivity with the nuclei in a small number of cardiac cells from all suicidal carbon monoxide poisoning victims, although reactivity of anti SIRT1 was weaker than that observed with anti CIRBP and RBM3. No reactivity with anti SIRT1 in the nuclei was detected in the myocardium from an individual who died by suicidal burning and cardiac tamponade. The frequency of SIRT1 reactivity in the nuclei in the myocardium was lower than that in the hypothermic death cases [4]. Anti SIRT1 also showed granulated reactivity in the cytoplasm of the colliquative myocytolysis and/or hypereosinphilic cells located far from contraction bands and diffuse weak reactivity in the cytoplasm in the cells containing contraction bands in the hypothermic death, suicidal carbon monoxide poisoning, and asphyxia cases. The number of nuclei with anti CIRBP, RBM3, and SIRT1 reactivity seemed to correlate with the number of the remaining granular cells in the cerebellum, that is, the number of nuclei were higher in the hypothermic victims and decreased in carbon monoxide or asphyxia, and house fire victims, and were lowest in the suicidal burn cases.

Although anti HSP70 showed granulated reactivity only in the cytoplasm in the colliquative myocytolysis and/or hypereosinphilic cells in all cases examined, irrespective of level of COHb, the reactivity was very intense in the suicidal CO poisoning, weak in the house fire, and very weak in the suicidal burn victims. Anti e-NOS showed intense and granulated reactivity in the cytoplasm of the colliquative myocytolysis and/or hypereosinphilic cells, cardiac cells, and small vessel endothelial cells in all individuals of suicidal CO poisoning but no reactivity was observed in the nucli. The intensity of reactivity by e-NOS was the similar to that of anti HSP70 (Fig. 3).

Anti AIF, HIF-1α, VEGF, and P53 antibodies showed weak or moderate reactivity in the cytoplasm of the colliquative myocytolysis and/or hypereosinphilic cells from all individuals with high levels of COHb (Fig. 3). These antibodies showed also weak reactivity in the cytoplasm of the myocardium obtained from suicidal burn cases and in the hearts with thermal damaged myocardium. Anti VEGF and e-NOS antibodies showed good reactivity with blood vessel endothelial cells in the

heart from all cases.

No reactivity of anti C9 and C4 was observed in the myocardium from all individuals examined, although blood vessel endothelial cells showed good reactivity and the myocardium from the cardiac tamponade case showed good reactivity.

In comparing the cases, the reactivity of anti CIRBP, RBM3, and SIRT1 was intense in hypothermic death. Anti SIRT1 stained the cytoplasm of the asphyxia victim, and anti RBM3 showed some reactivity with the nuclei in the cardiac tamponade case. The reactivity with anti HSP70, ALF, HIF1α, VEGF, eNOS, and p53 basically resembled that of the carbon monoxide poisoning and related death cases, showing different intensity among the antibodies and individuals.

DISCUSSION

The process of heart failure and death in individuals examined in this study was different from that of cardiac infarction. In cardiac infarction, death occurs mainly after insufficiency of cardiac contraction caused by focal or local ischemic damage caused by occlusion or obstruction of the coronary arteries. In contrast, in both hypothermic death and carbon monoxide poisoning death, the heart is globally affected by the lack of oxygen and by the increase in carbon monoxide. Furthermore, heart muscle contraction may decline gradually. Based on our study, the staining characteristics of CIRBP, RBM3, and SIRT1 in the heart tissues from coronary infarction were distinguishable from those in carbon monoxide and hypothermic death cases. In our study, these antibodies stained the nuclei of the myocardium from suicidal carbon monoxide poisoning, hypothermic death, and asphyxia individuals, although the staining intensity was different and independent.

CIRBP and RBM3 were originally described as mild hypothermic stress proteins. These antigens were clearly detected in the heart tissue from hypothermic death individuals in our previous study [4], and these antigens were also detected in the heart tissue from carbon monoxide poisoning and from cardiac tamponade cases in the present study, and in the brain from self-strangulation death cases [17]. These results together with those of Wellmann *et al.* [18] indicate that these antigens were present in the patients with hypothermia and with hypoxia. HIF1α regulates the genes encoding the angiogenesis factor VEGF, inducible nitric oxide synthase, lactate dehydrogenase, and erythropoietin, which trigger the cascade of angiogenesis [19]. These genes were not detected in the nuclei of cardiac cells in this study. Since Lee *et al.* [20] reported that the HIF1α protein was not present in either non-infarcted or non-ischemic myocardium, it seems that the nuclei of cardiac cells from carbon monoxide poisoning death showed no or very weak reactivity with anti HIF1α, VEGF, and e-NOS antibodies, although reactivity with CIRBP, RBM3, and SIRT1 was detected. The results indicate that the HIF1α antigen is present after cell necrosis and that the transcription of CIRBP, RBM3, and SIRT1 seems to occur earlier than the cell necrosis. Anti C9 and C4 showed no myocardial reactivity and clear reactivity with blood vessel endothelial cells. These two antibodies showed intense reactivity only in an infracted cardiac lesion, and the reliability of these antibodies to detect the lesion caused by carbon monoxide intoxication [2] was previously reported.

The results obtained in the present study suggest that antigens, such as CIRBP, RBM3, and SIRT1, may become useful temporal markers of global hypoxic or ischemic injury in the myocardium.

The number of nuclei with anti SIRT1 reactivity in the myocardium was lower than that observed in hypothermic death cases [4]. Anti SIRT1 also showed granulated reactivity in the cytoplasm of the hypereosinophilic cells located far from the contraction bands and diffuse weak reactivity with the cytoplasm in the cells around the contraction bands in the victims of suicidal carbon monoxide poisoning. Weak reactivity of this antibody was observed in individuals who died in house fires and from suicidal burning. We have also found that the reactivity of anti SIRT1 was very weak in the neuronal cells in individuals who died due to self-ligature strangulation Weak reactivity was observed only in the nuclei of the neuronal cells in the basal ganglia and in the hippocampal granular cells [17]. The reasons for the different expression patterns among CIRBP, RBM3, and SIRT1 in organs and in death situations remain to be resolved. Although the postmortem intervals of the individuals examined in this study were different (Table 1), the reactivity pattern with the antibodies used in this study showed no remarkable differences. This study also indicates that the cells once affected by stress and hypoxia, develop and activate signal pathways to prevent tissue damage, and the expressed antigens during the agonal period may be maintained after cell death and the postmortem interval.

References

1. Fineschi V, Agricola E, Barrodi C, Bruni G, Cerretani D, Mondillo S, Parolini M, Turillazi E, Myocardial findings in fatal carbon monoxide poisoning: a human and experimental morphometric study, Int J Legal Med 2000; 113: 276-282.

2. Fracasso T, Pfeiffer H, Michaud K, Koehler H, Sauerland C, Schmeling A, Immunohistochemical expression of fibronectin and C5b-p in the myocardium in cases of carbon monoxide poisonong. Int J Legal Med, 2011; 125: 377-384.
3. Morita S, Furukawa S, Wingenfeld L,Nishi K, Pathological findings of myocardium in carbon monoxide poisoning are basically similar to those in hypothermic deaths. Anil Aggrawal's Internet Journal of Forensic Medicine and Toxicology, 2013; 14(2), Available from: http://www.anilaggrawal.com/ij/vol_014_no_002/papers/paper012.html
4. Furukawa S, Wingenfeld L, Sakaguchi I, Nakagawa T, Takaya A, Morita S, Yamasaki S, Nishi K, Histopathological characteristic 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's Internet Journal of Forensic Medicine and Toxicology, 2013; 14(1), Available from: http://www.anilaggrawal.com/ij/vol_014_no_001/papers/paper004.html
5. Katsumata Y, Aoki M, Sato K, Suzuki O, Oya M, Yada S, A simple pectrophotomery for determination of carboxyl-hemoglobin in blood, J Forensic Sci. 1982; 27: 928-934.
6. Morita S, Furukawa S, Takaya A, Sakaguchi I, Okunaga H, Nakagawa T, Wingenfeld L, Nishi K, Usability of histological assessment of cerebellar granule cell layer regardless of postmortem interval. J Forensic Research, 2013; 4:180. doi:10.4172/2157-7145.1000180
7. Nishiyama H, Itoh K, Kaneko Y, KIshishita M, Yoshida O, Fujita J., A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth, J Cell Biol 1997; 137: 899-908.
8. Danno S, NIshiyama H, Higashiguchi H, Yokoi H, Xue J H, Itoh K, Matsuda T, Fujita J, Increased transcript level of RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress". Biochem. Biophys. Res. Commun. 1997; 236: 804–807.
9. Chen R, Dioum E M, Hogg R T, Gerard R D, Garcia J A, Hypoxia increases Sirtuin 1 expression in a hypoxia-inducible factor-denpendent manner, J Biol Chem, 2011; 286:13869-13878.
10. DE Maio A, Heat shock proteins: facts, thoughts and dream. Shock, 1999; 11: 1-12.
11. Chen R, Dioum E M, Hogg R T, Gerard R G, Garcia J A, Hypoxia increases sirtuin 1 expression in a hypoxia-inducible factor-dependent manner, J Biolo Chem, 2011; 286: 13869-13878.
12. Ferrara N, Davis-Smyth T, The biology of vascular endothelial growth factor. Endocr Rev, 1997; 18: 4-25.
13. Southan GJ, Szabó C, Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem. Pharmacol. 1996; 51 (4): 383–394.
14. Joza N, Susin SA, Daugas E, Stanford W L, Cho S K, Li C Y, Sasaki T, Elia A J, Cheng H Y, Ravagnan L, Ferri K F, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong Y Y, Mak T W, Zuniga-Pfluecker J C, Kromer G, Penninger J M, Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 2001; 6828: 549-554.
15. Collard CD, Lekowski R, Jordan J E, Agah A, Stahl G L, Complement activation following oxidative stress, Mole Immnolo. 1999, 36: 941-948.
16. Jenkins CP, Cardona D M, Bowers J N, Oliai B R, Allan R W, Norman S J, The utility of C4d, C9, and Tropnin T immunohistochemistry in acute myocardial infarction. Arch Pathol Lab Med. 2010, 134,256-263.
17. Furukawa S, Sakaguchi I, Morita S, Nakagawa T, Takaya A, Wingenfeld L, Nishi K, Suicidal ligature strangulation without an auxiliary mechanism. Reports of two cases with a cotton or a T-shirt and staining results of the brains using anti HSP-70, CIRBP, RBM3, HIF-1 alpha, SIRT-1 and p53 antibodies. Rom J Legal Med. 2013, 21, 9-14.
18. Wellmann S, Buehre 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.
19. Sharp F K, Bernaudin M, HIF1 and oxygen sensing in the brain, Nature Reviews Neuroscience, 2004; 5: 437-448), was not detected in the nucleus of cardiac cells in this study. Since Lee *et al.* (Lee S H, Wolf P L, Escudero R, Deutsch R, Jamison S W, Thistlewaite R A, Early expression of angiogenesis factors in acute myocardial ischemia and infarction, New Eng J Med. 2000, 342, 626-633).
20. Lee S H, Wolf P L, Escudero R, Deutsch R, Jamison S W, Thistlewaite R A, Early expression of angiogenesis factors in acute myocardial ischemia and infarction, New Eng J Med. 2000, 342, 626-633).