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원저

흰쥐 혈관 내에 존재하는 봉한관의 호은성 섬유

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• 목적 : 흰쥐의 혈관 내 실 모양 구조물의 조직학적 특성을 규명하여 봉한관과의 일치 여부를 조사하였다.
• 방법 : 실 모양 구조물의 조직학적 특징을 관찰하기 위해 헤마톡실린-에오진 염색과 고모리(Gomori)의 호은성 섬유 염색 방법을 사용하였다.
• 결과 : 혈관 내 실 모양 구조물 내에 호은성 그물 섬유와 진하게 염색된 타원형 혹은 막대 모양 핵이 존재함을 밝힐 수 있었다.
• 결론 : 혈관 내 실 모양 구조물이 혈관 내 봉한관임을 보이는 강력한 조직학적 근거를 얻었다.

Argyrophilic Fibers of Intravascular Threadlike Structures in Rat

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ABSTRACT

Objective We took intravascular threadlike structures from rat aortas to investigate their histological characteristics consistent with the intravascular Bonghan duct.

Methods Gomori's silver impregnation method, in addition to routine hematoxylin and eosin staining, was applied to demonstrate the characteristic feature of the intravascular threadlike structures.

Results These two staining methods clearly showed that the intravascular threadlike structures had unique features of argyrophilic reticular fibers and heavily stained oval or rod-shaped nuclei in them.

Conclusion The results are strong evidences for identifying threadlike structure as the intravascular Bonghan duct.

Key words *Acupuncture meridian, Argyrophilic fiber, Bonghan duct, Fluorescence stereomicroscope, Intravascular threadlike structure*

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I. INTRODUCTION

Acupuncture treatment has been widely accepted as an alternative medical practice, and the scientific investigation of its mechanism has received attention from various fields[1]. Currently neurophysiological theories are considered to be the most viable in regard to the anesthetic effects of, and pain control by, acupuncture treatments[2,3]. According to the neurophysiological viewpoint, there are no specific anatomical structures at the acupoints or acupuncture meridians, and the effects of acupuncture should be understood in terms of known structure, such as the system of nerves, hormones, and so on. However, there are experimental evidences which suggest the existence of a separate circulatory system of acupuncture meridians that are different from the nervous system, or blood and lymphatic vessels: radio-isotope tracing[4], low-electrical impedance[5] and thermal transmission along the meridians[6] and more CO_2 production[7,8] and biophoton emission[9] at the acupoints. Yet, various studies have shown no definite evidence for anatomical or histological structures corresponding to the acupoints or meridians[10-12].

It was Bonghan Kim who published his group's findings in early 1960's on the substance of the acupuncture points and meridians, a new anatomico-histological system in the living body[13-15]. It was a novel circulatory network entirely different either from the nervous system or the blood and lymphatic vessels. His group clarified the histological microscopic composition of a structure(Bonghan corpuscle) found at the acupoints and a tubular structure(Bonghan duct; BHD) which connected the Bonghan corpuscles and corresponded to meridians. The Bonghan corpuscles and BHDs were distributed not only in the superficial layer of the skin, that corresponded to the acupoints and meridians, but in the profound subcutaneous tissues, in the blood and lymphatic vessels and around the internal organs as well. Most surprising in the anatomical sense was the existence of BHDs within arteries and veins in an isolated manner, not adhering on the inner walls of vessels. Another discovery was the flow of specific liquid circulating along the BHD. The liquid contained a large amount of granules that were composed of DNA, whose physiological roles would be, in modern terms, cell-therapy, and the granules would be called toti-potent adult stem-cells[16,17].

Unfortunately he left only one English publication[14]. To make things worse he did not disclose the staining material and the method that had led him to observe the claimed structures in the subjects such as rabbits, which would have been an essential step if other people were to try and confirm his results. Thus despite intense efforts in the far eastern countries no one has ever been able to confirm the claimed structures for almost forty years, and his findings have been forgotten except by the Japanese anatomist Fujiwara and his associates who were able to reproduce much of Kim's works[18,19]. However his confirmation has also been neglected. Only very recently a new method of perfusion has been introduced to find the BHD inside the blood vessels of rats, mice, and rabbits. Indeed threadlike structures from the major arteries and veins were observed by careful techniques of slow perfusion with high-density dextrose solutions[20]. The existence of threadlike structures inside blood vessels was a novelty in current anatomical knowledge, but it has required further examinations to prove that they are indeed the BHDs. For this purpose we contrived a fluorescence method to observe nuclear distributions in the threadlike structure by staining with acridine-orange. Confocal laser scanning microscopic images of the stained specimen revealed the characteristic features of the BHD distinctly: the nuclei were long and rod-shaped, 10-20 μm in length, and they were aligned to form

broken-lines[21]. These features were in good agreement with Kim's original work[15].

One of the most important features to identify the BHD is the argyrophilic fibers that constitute intertubular material of the BHD, which is itself a bundle of multiple tubules. In this paper we present the silver impregnation technique as a method to reveal these argyrophilic fibers in the intravascular threadlike specimens that we have obtained from the abdominal aortas of rats.

The existence of threadlike structures inside blood vessels is astonishing in view of western anatomy. Despite its importance and wide interest no one could either validate or disprove Bonghan Kim's claims. The question of its truth and falsehood remained unresolved for many years. One of the main reasons to make the problem difficult was that Kim kept the method secret and stated the results only. In this article we introduce and state concretely our own methods to observe the intravascular threads, and the techniques of silver impregnation to reveal reticular fibers in the Bonghan ducts. This is a major step forward in establishing the existence of threadlike tissues inside blood vessels, and thus leading to full investigation of the third circulatory system that will be an epoch making achievement in western anatomy.

II. MATERIALS AND METHODS

Sprague-Dawley rats of 6-8 weeks that were obtained from the Laboratory Animal Center of Seoul National University were used in this study. The animals were housed in a constant temperature-controlled environment(22±3℃) with 55±5 % relative humidity. All the animals were fixed at a 12 hr light-dark cycle, and had ad *libitum* access to food and water. Procedures involving animals and their care conformed with institutional guidelines, which were in full compliance with current international laws and policies(Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

The rats were anesthetized with urethane(1.5 g/kg) administered intraperitoneally, and all surgical procedures were performed under general anesthesia. Under deep anesthesia, the abdominal sides of the rats were incised, and the stomach, intestines and perivascular fats were moved to one side, and then their abdominal sides were also opened to isolate the abdominal aorta and caudal vena cava. We softly squeezed the target blood vessel between thumb and middle finger and quickly cut and took the surrounding connective tissues off the body to preserve the specimen fresh. We made use of a simple technique to isolate the intravascular Bonghan duct(IBHD) embedded in fibrin from the blood vessel. Even though this method looks simple and unsophisticated, it is very effective in sampling the specimen in a fresh condition. The isolated blood vessel was dipped into a Petri dish containing phosphate buffered saline(PBS, pH 7.4) and the connective tissue attached to the blood vessels was carefully removed in the PBS. Further delicate operations were done on a black rubber plate, both for obtaining a better contrast image and also for keeping the samples from drying. On a black rubber plate the abdominal aorta with its surrounding connective tissues was longitudinally dissected a little bit on the upper part of the vessel wall by micro scissors. An edge of the fibrin string that enshrouded a BHD was found in the middle of the intravascular space. As will be shown later the endothelial layer was intact except the dissection. Longitudinal dissection was continued to the end of abdominal aorta. The acridine-orange fluorescence method[21] was applied to confirm the BHD containing fibrin string, and these processes were done under a fluorescence-stereomicroscope(Fig. 1B).

Immediately after taking threadlike structures embedded in varying amounts of fibrin we fixed

them in 10 % neutral buffered formalin for cryosection on the next day. Gomori's silver impregnation technique was employed to demonstrate argyrophilic reticular fibers, which is a characteristic feature of the intravascular Bonghan duct[22]. Hematoxylin and eosin staining was performed to discriminate nuclei and cytoplasm in addition to reticular fibers. We concurrently stained the abdominal aorta, in order to demonstrate that the endothelium of the abdominal aorta from which the specimen was obtained was not damaged, that is, it was intact.

III. RESULTS

In the previous works[20-22], the intravascular threadlike structure embedded in fibrin was obtained from the abdominal aorta after dextrose perfusion(FIG1A). In a series of work for confirming Bonghan Kim's claim we could improve the sample taking method. Based upon the previous works we devised a new method not to use dextrose perfusion. For the purpose of comparing the two methods(perfusion and non-perfusion) we present two figures(FIG 1A and FIG 1B). As described in the materials and method section the sample of a fibrin string containing an intravascular Bonghan duct was taken from the piece of the abdominal aorta which was cut off from the body. The sample was searched under a fluorescence stereomicroscope, and the Bonghan duct looked brighter than the fibrin part after staining with acridine orange(FIG 1B). Even though FIG 1A shows more impressively the sample taking procedure, it requires much more delicate and time consuming operation of slow perfusion by a skillful surgeon. Furthermore it did not reveal the enshrouded Bonghan duct while FIG 1B showed directly the presence of the Bonghan duct beside the fibrin.

During a series of experiments with varying techniques we obtained many samples of the threadlike structures from several blood vessels(the caudal vena cava, abdominal aorta, aorta, common iliac vein, femoral vein). We measured the length of the nuclei, the separation distance between two neighboring nuclei on an aligned line, and the diameter of the threadlike structure. These data are shown in Table 1. The subjects are ordered according to the dates of the experiments we performed.

The average length of the rod-shaped nuclei was $18.3 \pm 5.2\ \mu$m, and this value was nearly uniform throughout the samples. We notice that the separation distances are about two times of nuclei-length, and the thickness of the threadlike structure vary widely depending upon the blood vessels and the physiological states of the subjects. The average thickness and the standard deviation of the threadlike structure were $44.9 \pm 30.4\ \mu$m and the thickest and thinnest ones are $13.6\ \mu$m and $134.9\ \mu$m, respectively. The number of samples taken from each subject depended upon surgeons' experimental skills, rather than the inherent property of the subject.

The string of fibrin containing several nuclei of the threadlike BHD was stained by hematoxylin and eosin(FIG 2A). In this picture, fibrin was randomly distributed, having a pale pink color and eosin stained red blood cells were clearly seen in fibrin nets(FIG 2A). Gomori's silver impregnation technique showed clearly that there were argyrophilic reticular fibers in the string of fibrin and several nuclei heavily impregnated by silver in the bundle of the fibers(FIG 2B) as well as stained by hematoxylin.

In order to rule out the mistake that the threadlike structure might be an artifact that had peeled off from the endothelium of the blood vessel, the status of the internal layer of the abdominal aorta using the same hematoxylin and eosin staining was intact(FIG 3).

IV. DISCUSSION

Our previous works[20-22] on intravascular Bonghan ducts(IBHD) showed that there were threadlike structures inside the blood vessels of rats, a fact which no one had previously imagined. This exceedingly thin threadlike structure has a characteristic feature in its nuclear shapes and the arrangements of its nuclei, which is in good agreement with the former discoverer, Bonghan Kim's description[15]. However, our previous works need further work to demonstrate the detailed features of IBHD, especially in sectional image which is considered important in histological work. For this purpose we designed a new protocol to demonstrate one of the most distinctive features of IBHD, that is, the argyrophilic fibers which constitute the IBHD. The new protocol is to reveal that the IBHD is not an artifact but a genuine intravascular structure based upon two histological facts: one fact is that the vascular endothelium has no argyrophilic fiber and another is that the IBHD has a lot of argyrophilic fibers as a connective tissue and nuclei, in contrast to the endothelium.

The IBHDs were hard to observe because of their transparency and thinness, and thus it is difficult to take the target sample on the incised blood vessel under an ordinary light stereoscopic microscope. In the current experiment we used a fluorescence stereoscopic microscope(FSM) to observe and to locate, *in situ*, the desired intravascular threadlike structure. The specimen looked very distinct from a blood vessel or a blood clot under the fluorescence stereomicroscope after staining it with 0.1 % acridine orange(AO). Under the FSM string-like structure of a blood clot appeared darker than the IBHD and the endothelium of a blood vessel(FIG 1B). Blood clots consisted of red blood cells and fibrin that had no nuclei and were not stained with AO, a DNA-staining fluorescence dye. In order to confirm this we further examined the sample that was put on a slide with a confocal laser scanning microscope, to see whether this threadlike structure had rod-shaped nuclei as described in our previous papers[21,22](FIG 4A and FIG 4B) as well as Bonghan Kim's original work[13-15].

As expected there was a great amount of fibrin in the string structure enshrouding the IBHD. We hypothesized that the IBHD acts as a long and floating seed that forms a thick string with a varying amount of fibrins around itself in an emergency situation, such as the surgical injury of blood vessels.

FIG 2A shows the string structure stained by hematoxylin and eosin. In this picture fibrin of pale pink color is randomly distributed with red and white blood cells. The pink color of fibrin and red blood cells is to be attributed to their eosinophilic properties[22]. And white blood cells are stained by hematoxylin in addition to eosin, owing to their nuclei. FIG 2A also shows oval or rod-shaped nuclei, heavily stained by hematoxylin, and their nucleoli look obscure. The nuclei heavily stained by hematoxylin with hardly discernible nucleoli are what was also reported by Kim[15].

The string structures that are usually formed during ordinary surgery have long been overlooked, being considered as just fibrin only. This overlooking is due to the similarity of the fibrin and the IBHD under ordinary light microscope, such that discerning one from the other is extremely difficult. In order to prove that this extra structure is the IBHD, we examined them further to find out whether there are reticular fibers among these oval or rod-shaped nuclei as Bonghan theory dictated. According to the theory, there must reside reticular fibers among these oval-shape nuclei.

FIG 2B shows the silver impregnation image of an intravascular threadlike structure embedded in fibrin from the abdominal aorta of a rat. Gomori's silver impregnation technique showed clearly that there were argyrophilic reticular fibers in the threadlike structure, which would have been mistaken as fibrin with blood cells captured in it

without careful and serious examination like ours. The reticular fibers co-existing with oval-shaped nuclei were heavily stained by hematoxylin. Such a feature is a characteristic mark of a Bonghan duct[15]. This figure also showed that the nuclei in the threadlike structure were strongly impregnated by silver as well as stained by hematoxylin. In the viewpoint of histology, it is well known that reticular fibers are derived from the fibrocytes, what is called, reticular cells at the same site. This histological aspect implies that nuclei heavily stained by hematoxylin and simultaneously impregnated by silver are the nuclei of the reticular cells. However, further immunostaining studies using reticular fiber antibodies would more firmly establish this implication.

After investigating the threadlike structure from an incised vessel with both hematoxylin and eosin staining and silver impregnation, we examined the endothelium of the blood vessel from which the threadlike structure was taken using the same Gomori's silver impregnation. This additional experiment was performed to eliminate any suspicion that the structure might be an artifact that had peeled off from the endothelium. FIG 3 shows that the endothelium in the rat abdominal aorta, where the IBHD sample was taken, was intact. Thus the threadlike IBHD is clearly different from the endothelium of a blood vessel. Through such thorough procedures we were able to eliminate any chance of artifacts originating from blood vessels.

The above results revealed that the threadlike structures afloat inside blood vessels are composed of argyrophilic fibers. Our finding is another step in our series of works[20-22] towards the rediscovery and establishment of Bonghan theory, which has been forgotten for almost 40 years.

The reason for this long negligence was the lack of substantiating data to support the validity of the Bonghan theory. The only confirmation was by Fujiwara[18,19] which did not get much attention either. Very recently, however, rediscovery of his claims was seriously performed. After the work on the intravascular threadlike structure by Lee et al.[21], there appeared three independent groups to report about the Bonghan duct on the surfaces of internal organs of rats and rabbits[24-27]. In addition, Feulgen reaction study revealed the flow of Bonghan granules that contain DNA[28]. These recent results provided the long-sought evidences for the Bonghan theory. Even *in vivo* visual demonstrations of the intravascular Bonghan ducts and corpuscles are achieved[29,30]. Thus the investigations like the current argyrophilic study that would be deeply related to the physiological functions of the novel structure[31] are more worthwhile here after.

V. ACKNOWLEDGEMENTS

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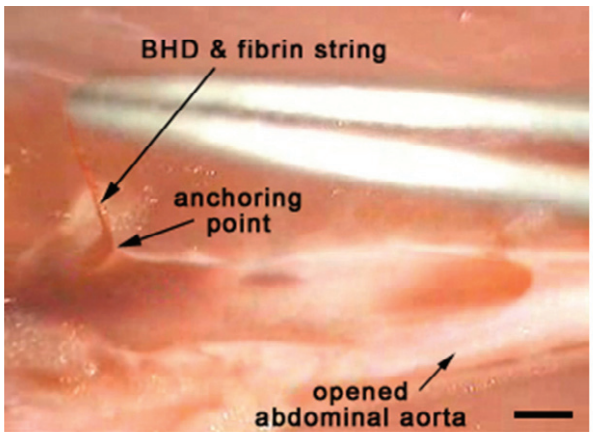

FIG. 1A. The abdominal aorta of a rat is opened, and a string of fibrin that contains pieces of the broken intra-vascular threads is held with a forceps. Notice that the string is anchored on the left upper vessel wall. This anchoring position had not been systematically studied because the thread structures were usually broken during the surgery-perfusion-searching processes. Scale bar = 2 mm.

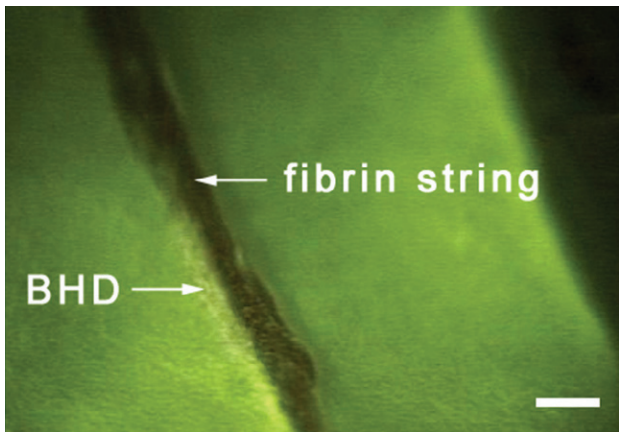

FIG. 1B. Fluorescence stereomicroscope image of the intravascular Bonghan duct that looks bright and locates at the left hand side lower part of the fibrin string that looks dark and thick at the middle line. The background is an inner vessel wall of the abdominal aorta. Scale bar = 500 μm.

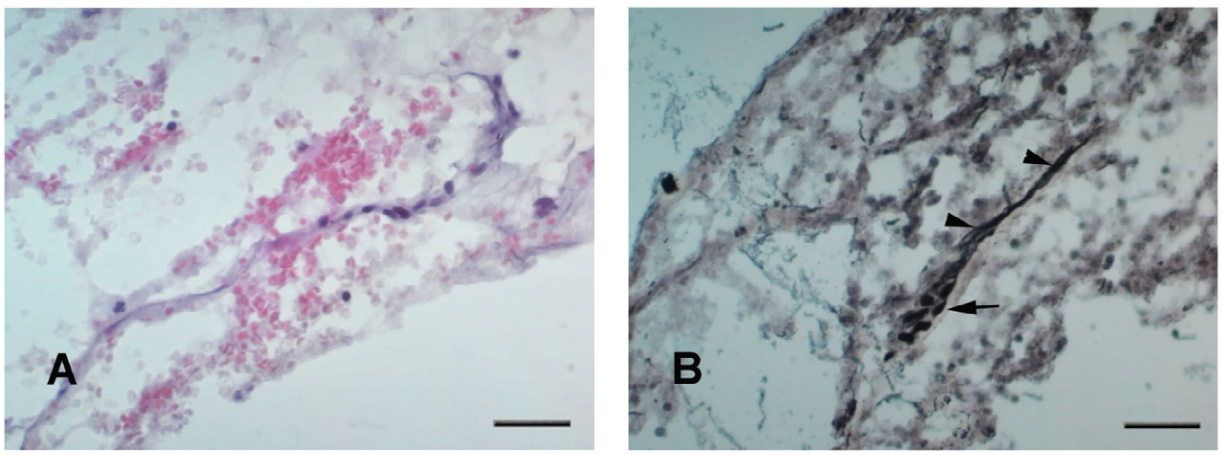

FIG. 2. Photomicrogram of the threadlike structure using hematoxylin and eosin staining (A). The threadlike structure using silver impregnation (B): argyrophilic reticular fibers(arrowheads) and oval-shaped nuclei(arrow) are seen. Scale bar = 30 μm.

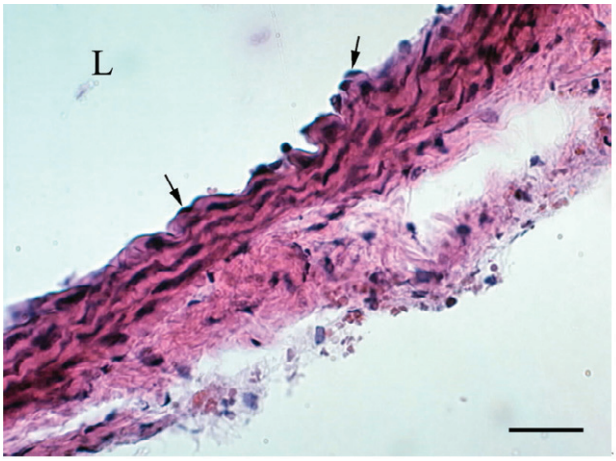

FIG. 3. Photomicrogram of the cross-sectioned abdominal aorta using hematoxylin and eosin staining. The endothelial layer including endothelial cells(arrows) of lumen(L) was intact. The threadlike structure was taken in the lumen of this blood vessel. Scale bar = 30 μm.

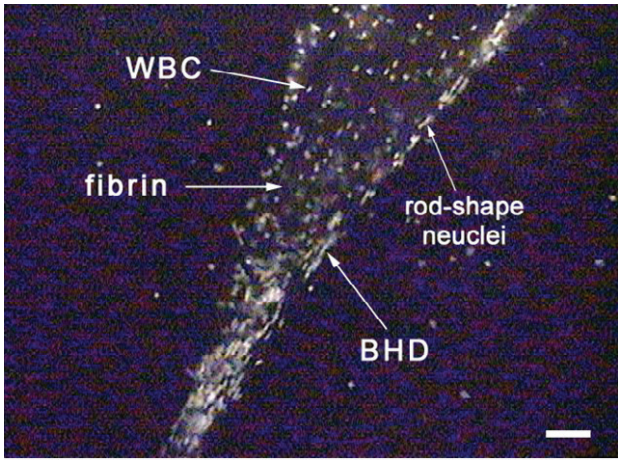

FIG. 4B. The threadlike structure with enshrouding fibrin was observed with acridine orange fluorescence method. The scattered dotted points were white blood cells, and the long rod-shaped nuclei were from the threadlike structure. This method clearly distinguishes fibrin from the Bonghan duct. Scale bar = 50 μm.

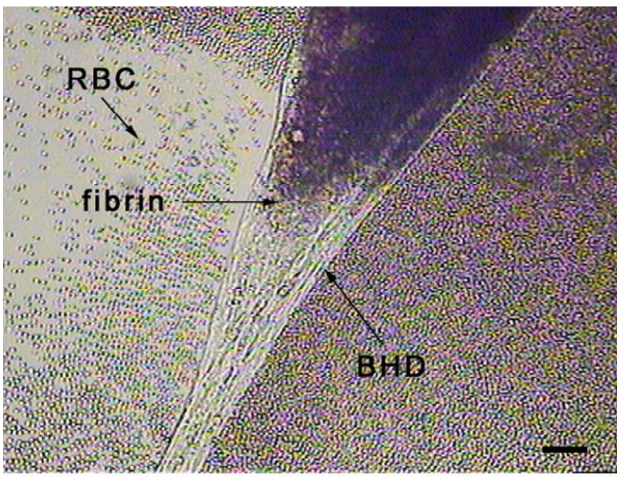

FIG. 4A. The threadlike structure with enshrouding fibrin was observed on a slide using a differential interference contrast microscope. The fibrin and the Bonghan duct were hardly distinguishable and red blood cells were scattered around. Scale bar = 50 μm.

Table 1. Size data of the intravascular threadlike structures from nineteen rats

Subject			Blood vessel where sample is taken	l(μm)	s(μm)	D(μm)
number	sex	Weight(g)				
1	M	420	CV	21.9	39.8	26.8
			CA	20.1	40.1	104.5
			CA	19.3	32.6	44.5
			AO	13.3	34.4	35.0
2	F	260	CV	16.4	39.0	53.5
3	F	300	CV	16.6	24.5	40.1
4	M	430	CV	23.1	41.6	39.4
5	M	470	IV	14.8	U	55.8
			IV	28.7	54.9	23.2
6	M	500	IV	18.6	48.1	33.5
			CV	24.6	53.1	40.5
7	M	300	CV	26.6	34.2	134.9
			IV	20.4	48.1	25.8
			IV	21.8	84.2	59.1
			IV	20.6	27.6	110.2
			FV	16.9	61.5	62.0
			CV	11.5	31.2	103.2
8	M	200	IV	11.0	15.5	35.7
			CV	9.55	31.3	57.4
9	M	200	CV	11.5	22.3	15.7
			CA	11.7	26.7	17.4
			IV	12.3	32.4	7.67
10	M	165	AO	16.2	39.6	18.8
			CA	14.0	44.9	25.7
11	M	205	AO	15.3	29.5	13.6
12	M	205	IV	12.2	33.4	37.2
13	M	190	IV	19.7	38.7	35.6
14	M	250	CA	17.5	31.7	14.9
15	F	310	CV	24.5	47.2	42.8
16	M	220	CA	20.5	40.2	72.5
17	M	240	CA	19.4	47.1	27.5
18	F	270	CA	25.3	50.2	49.6
19	F	210	CV	27.2	43.2	17.4
average				18.3	39.7	44.9
standard deviation				5.2	13.0	30.4

The abbreviation for blood vessels are as follows:

CV = caudal vena cava, CA = abdominal aorta, AO = aorta, IV = common iliac vein,

FV = femoral vein, l = the average length of nuclei of the sample

s = average distance between two neighboring nuclei aligned in a line

D =the diameter of the threadlike structure, U = not measured in the time of experiments

(Appendix)

Brief Summary of Bonghan Kim's Works

(1) Reference 13. Research about the realities of *kyungrak*

Acupuncture points are electrically singular: They have lower electrical resistance and higher potential compared with neighboring non-acupuncture points. There is a correlation between the movements of large intestines and electrical stimulations at the Zusanli points(ST 36).

Acupuncture meridians are anatomically real structures as small ducts with a bundle of tubules. There are corpuscles of oval shape at acupuncture points.

(2) Reference 14. On the *kyungrak* system

Kyungrak system is a new circulatory system that consists of Bonghan corpuscle and Bonghan ducts which are distributed subcutaneously and inside of the body. There are also branches of the net both inside and outside of blood vessels. There flows Bonghan liquid through the Bonghan ducts, and there are DNAs in the liquid.

(3) Reference 15. Theory of *kyungrak*

The Bonghan system is composed of several subsystems: Intravascular Bonghan ducts(BHDs) inside blood and lymph vessels; Organ-surface BHDs on the surfaces of internal organs; Extravascular BHDs in the outside of vessel walls; Neural BHDs in nervous systems including brain; Inside-organ BHDs. The chemical components of the Bonghan liquid include hyaluronic acid, adrenalin, noradrenalin, mononucleotide and DNA.

Histological studies showed detailed structures of Bonghan corpuscles and BHDs. A BHD is composed of many small tubules as a bundle. The endothelial layer of each small tubule has rod-shaped nuclei whose length are about 15-20 μm, and they are heavily stained by hematoxylin. Between the small tubules of a BHD there are argyrophilic fibers. Inside the BHD there are many basophilic granules.

Radioisotope tracing of the Bonghan system using P^{32}, and electrical properties were also performed to confirm their independence from blood, lymph and neural systems.

(4) Reference 16. *Sanal* theory

In the Bonghan duct(BHD) flows granules containing DNA, which is named *'sanal'* that literally means 'vitalsome'. A sanal is about 1 μm, in spherical shape, surrounded by a thin membrane. It includes about a single chromosomal amount of DNA.

It is generated from normal cells by the process of micronucleation, and then circulates in the BHD. Its physiological role is to regenerate cells in damaged tissues. Its cultivation is possible using Bonghan liquid: *Sanals* becomes a cell.

(Present authors' remark: In modern terms sanal is a microcell which is possibly a source of stem cells that does cell therapy function. It is most surprising that Kim found the concept of microcells and stem cell in early 1960's much earlier than western biomedical society started similar studies.

Sanal is a kind of microcells which are extensively studied in cell fusion and cancer studies. Microcells are generated by chemical agents like colcemid, or in pathological conditions of tumor. The only difference between *sanal* and microcells seems to be the generation mechanism: *Sanal* is naturally produced, whereas microcells are produced artificially.

See for example: Buikis I., Harju L., Freivalds T., Origin of microcells in the human sarcoma cell line HT-1080. Analytical Cellular Pathology 1999;18;73-85.)

(5) Reference 17. *Sanal*/cell cycle of blood cells

New Hematopoietic theory is proposed based upon sanal theory. Blood cells are produced mainly

in the intravascular Bonghan corpuscles from the growing *sanals*.

(Present authors' remark: This theory is quite contrary to the current bone marrow hematopoietic theory. However, one should note the origin of hematopoietic stem cells is not firmly established and Bonghan theory could be a possible source of these adult stem cells.)