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Study on the action and mechanism of humic acids in Kaschin-Beck disease

Wang Weizhe[1], Yang Zhiwei[1] and You Shengzhi[1]

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Abstract— Humic acids, especially FA in fractions, contain more oxygen functional groups. In this experiment, on the basis of confirming the action of humic acids on KBD, what was studied is the biological effects of one of main oxygen functional groups, hydroxy group (–OH). The results indicate that inducing pathologic process of KBD, obviously decrease the GSH-Px activity and induce peroxidation membrane injury of tissue. The SOD activity increase in the tissues caused by oxygen functional groups showed that enhancing of free radical reaction should not be neglected.

Keywords: Kaschin-Beck disease; humic acids; oxygen functional groups; free radical; antioxidase.

INTRODUCTION

We have reported that the native degradation products of organic matter in the grain (deteriorated products) and the drinking water (humic acids) in KBD region may be the cause of KBD (Wang, 1990). Takezawa had once presented and demonstrated that humic acids was the causal agent of KBD and believed ferulic acid and *P*-camaric acid played important role in the cause of KBD (Takezawa, 1970). Humic acids which contained more oxygen functional groups such as carboxyl (–COOH) and hydroxyl (–OH) played very important role in the growth and development of plant (Schinitzer, 1972). When we found in studies that in the extracted fractions of humic acids from soil and drinking water from KBD region the injurious effect of fulvic acid (FA) on cultured chondrocytes was more than that of humic acid (HA), naturally our first thought was that the injurious effect of humic acids is related to higher oxygen content in FA and more oxygen functional groups of FA than those of HA (Wang, 1985; 1986).

STUDY ON CULTURED CHONDROCYTES

1. Under the condition of asepsis, the tibia and the femur were drawn from chick embryo of Leghorn, cultured for 12 days, digested, and then chondrocytes were scattered in epiphyseal cartilage by 0.25% trypsin. The medium consisted of mixture of 199 (Nissui) and

1. Department of Pathophysiology, Liaoning Institute of Basic Medicine, Shenyang, China.

equal RPMI 1640 (Serva), 10% newborn bovine serum, 100 Uml^{-1} penicillin, 100 μgml^{-1} streptomycin. Cells, at a concentration of 40×10^4 ml^{-1} medium, were poured into culture bottles with slides and were cultured at 37°C. Culture bottles kept sealed and stabilized in the course of culture. After cultured for approximately 40 hours, cells were observed by inverted microscope. The culture bottles with cells attached to the wall of bottle and good growth were divided randomly into several groups. Fresh medium replaced old one every other day. Cells were sub-cultured once and monitored for 14 days. At 3, 7 and 14 days of this experiment culture bottles were randomly chosen from every group. After removing the slides, we took histochemical assay, compared the alkaline phosphatase activity (ALP-ase, Goncori's method) and sulphate glucosaminoglycan content (SGAG, toluidine blue method). The results indicate that the chondrocytes of ferulic acid group, at a concentration of 50 ppm (2.6×10^{-4} mol), exhibit poor growth and the gathered round-cell groups (cartilage island) were hardly seen. More cells grew in the shape of shuttle or pod and were full of fatty drops in cytoplasm. The ALP-ase activity and SGAG content were lower than that of the control. At a raised concentration of 500 ppm (2.6×10^{-3} mol), the chondrocytes growing on the wall of bottle necrosed quickly and dropped off. The injured degree of chondrocytes of *p*—cumaric acid group was obviously more serious than that of ferulic acid group. At a concentration of 5ppm (3.0×10^{-5} mol), injury to cells was seen and the ALP-ase activity and SAGS content of the group was lower than that of ferulic acid group. At a concentration of 50 ppm (3.0×10^{-4} mol), more cells necrosed and dropped off. In most of the residual chondrocytes attached to the wall, cell bodies were small and thin, and nuclei and nucleoli were unclear, and cytoplasm were full of fatty drops, and the ALP-ase and SGAG content were gradually reducing. At a raised concentration of 500 ppm (3.0×10^{-3} mol), chondrocytes were totally broken up and necrosed and dropped off in about 3—5 days. From the results, we believe that the injurious effect of phenolic acid on chondrocytes were obviously shown at a concentration of 10^{-4} mol. The injurious effect of P-cumaric acid containing only a hydroxyl (—OH) on chondrocytes was obviously more than that of ferulic acid (Table 1).

2. The epiphysis extremity of fetal long bone in limbs were drawn from 3—4 months fetus in an induced artificial abortion and digested by 0.25% trypsin. The scattered chondrocytes were diluted into 4.0×10^4 cells ml^{-1} by Ham F12 medium (including 20% newborn bovine serum. 100 U ml^{-1} penicillin and 100μg ml^{-1} streptomycin). Thereafter, they were cultured and observed with the same method as chick-embryo mentioned above.

Three reagents used in this experiment were N-valeric acid, benzoic acid and 3, 4-dihydroxybenzoic acid. Their molecular weights were 102.3, 122.12 and 154.12, respectively. Benzoic acid and 3, 4-dihydroxybenzoic acid each have a benzene ring and the latter has two hydroxyls (—OH). Their concentrations used in this experiment were 1×10^{-5} —10^{-3} mol.

Table 1 Injurious effects of ferulic acid and P-cumaric acid on cultured chondrocytes

Group	Injury degree of chondrocytes, 14 days	SGAG content, 3 days	7 days	14 days	ALP-ase activity, 3 days	7 days	14 days
Control	−	−	++	+++	+	++	+++
Ferulic acid							
5 ppm	−	+	++	+++	+	++	+++
50 ppm	++	+	+	++	+	+	++
500 ppm	+++	±	(−)	(−)	(−)	(−)	(−)
p-sumaric acid							
5 ppm	±	+	+	++	+	++	++
50 ppm	++	+	+	±	+	+	+
500 ppm	+++	−	(−)	(−)	(−)	(−)	(−)

Note: (−) shows that cells on slide fully dropped off

Table 2 Biological effects of organic acids with different structures on human-embryo chondrocytes

Group	Control	*n*-valeric acid 10^{-5}	10^{-4}	10^{-3}	Benzoic acid 10^{-5}	10^{-4}	10^{-3}	3, 4-dihydroxybenzoic 10^{-5}	10^{-4}	10^{-3}
Cell growth										
3 days	++	+++	++	+	+++	++	+	++	+	±
6 days	+++	++++	+++	+	++++	+++	+	++	++	±
SGAG synthesis										
3 days	+	+	+	+	+	+	+	+	+	±
6 days	++	++	++	+	++	++	+	++	+	±

At 3 and 6 days of this experiment, the growing condition and the ability of cells to synthesize SGAG were observed. The results were shown in Table 2. At a concentration of 1×10^{-5} mol, the three reagents had obvious effect of promoting growth and fission of chondrocytes. The cells bodies became small and the cell density increased. Comparatively, the effects of N-valeric acid and benzoic acid were more than that of 3, 4-dihydroxybenzoic acid. When concentration of reagents were added to 1×10^{-4} mol, the effect of promoting growth and fission of cells as mentioned above disappeared. The 3, 4-dihydroxybenzoic acid test group began to exhibit the effect of inhibiting growth and fission of chondrocytes. When concentrations of reagents were raised to 1×10^{-3} mol, all of three reagents showed obvious

inhibiting and injurious effect on growth fission of chondrocytes. Cells became thin and shuttle in shape and more space existed among cells. The ability of cells to synthesize SGAG decreased. The changes were obvious in 3, 4-dihydroxybenzoic acid.

After 0.1 ppm Se along with reagents of organic acid was added to medium, the injurious effect of the reagent in a concentration of 1×10^{-3} mol on chondrocytes disappeared and the ability of cells to synthesize SGAG returned to normal level (Table 3).

Table 3 The actions of different organic acids with Se on cartilage cells

Group	Control	*n*-valeric acid + Se			Benzoic acid + Se			3, 4-dihydroxybenzoic acid + Se		
		10^{-5}	10^{-4}	10^{-3}	10^{-5}	10^{-4}	10^{-3}	10^{-5}	10^{-4}	10^{-3}
Cell growth										
3 days	+	+++	++	+	+++	++	+	+++	++	+
6 days	++	++++	+++	++	++++	+++	++	+++	++	+
SGAG synthesis										
3 days	+	+	+	+	+	+	+	+	+	+
6 days	++	+++	++	++	++	+++	++	++	++	++

We find from recounting above that despite with different structures, organic acid reagents at a lower concentration can improve growth and fission of chondrocytes. On the contrary, organic acids at a higher concentration may injure cells and inhibit growth and fission of cells. The injurious effect of organic acid on cells was closely related to the number of −OH group in its structure and had little relation to whether or not there was a benzene ring present in its structure. Simultaneously, we demonstrated that Se had an obvious protection from the injurious effect of chondrocytes caused by organic acids such as humic acid (Wang, 1987).

STUDY ON ANIMAL EXPERIMENT

The rats used in this experiment were Wistar strain and the body weight was $120 \pm 20\mu g$. Number of female and male rats was equal. FA, a main fraction of humic acids, was extracted from soil beside water-well as a drinking water supply to local inhabitants. The −OH groups in some FA were blocked by the method of acetylation provided by Schnitzer and Skinner (Schnitzer, 1965).

The experiment was conducted in two steps.

1. Experiments were divided into three groups: non-KBD region, KBD region and

Table 4 The effects of FA on GSH-Px, SOD activities and LPO content in blood of the rats in KBD region and non-KBD region

Group	GSH-Px activity		SOD activity		LPO content	
	$M \pm SD$	P	$M \pm SD$	P	$M \pm SD$	P
Control	29.43±1.68		213.1±89.3		2.49±0.28	
Non-KBD region	30.27±3.33	>0.05	400.9±83.1	<0.01	2.56±0.19	>0.05
KBD region	23.11±1.94	<0.01	439.9±188.3	>0.05	2.60±0.23	>0.05

Note: Enzyme activity value is expressed by units ml^{-1} blood; LPO content —nmol ml^{-1} blood

Table 5 The influence of FA on GSH-Px, and SOD activities and LPO content in tissues of the rats in KBD region and non-KBD region

Groups	GSH-Px activity		SOD activity		LPO content	
	$M \pm SD$	P	$M \pm SD$	P	$M \pm SD$	P
Heart						
Control	701.8±64.9		300.4±90.9		209.6±14.8	
non-KBD region	729.4±91.7	>0.05	515.9±169.9	<0.05	215.4±18.1	>0.05
KBD region	594.1±115.4	<0.05	599.8±159.4	>0.05	217.1±12.5	>0.05
Liver						
control	791.5±62.3		873.3±137.5		490.5±23.9	
non-KBD region	797.2±52.4	>0.05	1033.0±121.4	<0.05	534.8±17.2	<0.01
KBD region	715.4±61.5	<0.01	1130.0±97.9	>0.05	556.6±19.5	>0.05
Muscle						
control	110.4±18.9		261.9±106.3		168.9±9.9	
non-KBD region	126.9±25.7	>0.05	443.1±159.4	<0.05	173.9±11.5	>0.05
KBD region	87.8±22.9	<0.01	524.1±86.7	>0.05	175.8±9.5	>0.05

Note: Enzyme activity value is expressed by units g^{-1} wet weight tissue; LPO content by nmol g^{-1} wet weight tissue

control group. Every group used ten rats. Beizen FA (non-KBD region in Liaoning Province) and Tianshui FA (KBD region in Gansu Province), by taking a dose of 100 μg/g body weight, were injected into the rat abdominal cavity of non-KBD region group and KBD region group, respectively. The same amount of physiological saline solution was injected into the rat abdominal cavity of the control group. At 72 hours after injecting, bloods in tail and heart, liver, gastrocnemius muscle were drawn and examined for antioxidative enzyme-GSH-Px and SOD activities and LPO content. The comparisons were made between KBD region group and non-KBD region group, and between non-KBD region group and control

Table 6 The influence of −OH-blocked and Se-supplement on changes in GSH-Px and SOD activities and LPO content in rat blood caused by FA in KBD region

Group	GSH-Px activity		SOD activity		LPO content	
	$M \pm SD$	P	$M \pm SD$	P	$M \pm SD$	P
KBD region	23.23 ± 1.53		336.4 ± 66.7		2.56 ± 0.09	
−OH-blocked	30.83 ± 1.47	< 0.01	268.2 ± 32.6	< 0.05	2.56 ± 0.28	> 0.05
Se-supplement	30.88 ± 3.57	< 0.01	326.6 ± 43.2	> 0.05	2.60 ± 0.29	> 0.05

Table 7 The influence of −OH-blocked and Se-supplement on change in GSH-Px and SOD activities and LPO content in the tissues caused by FA in KBD region

	GSH-Px activity		SOD activity		LPO content	
	$M \pm SD$	P	$M \pm SD$	P	$M \pm SD$	P
Heart						
KBD region	604.1 ± 122.5		501.2 ± 107.9		219.7 ± 12.7	
−OH-blocked	736.8 ± 140.4	< 0.05	447.3 ± 89.1	> 0.05	218.4 ± 13.7	> 0.05
Se-supplement	675.3 ± 121.8	< 0.05	495.5 ± 145.9	> 0.05	219.3 ± 13.2	> 0.05
Liver						
KBD region	723.1 ± 57.9		1474 ± 115		606.6 ± 48.9	
−OH-blocked	749.1 ± 55.9	< 0.05	1322 ± 129	< 0.05	491.1 ± 62.3	< 0.01
Se-supplement	794.8 ± 47.8	< 0.05	1498 ± 107	> 0.05	513.2 ± 59.7	< 0.01
Muscle						
KBD region	86.1 ± 26.6		147.9 ± 17.8		180.8 ± 12.5	
−OH-blocked	127.7 ± 27.7	< 0.05	124.2 ± 11.8	< 0.01	176.4 ± 14.5	> 0.05
Se-supplement	127.5 ± 29.7	< 0.05	162.5 ± 16.4	> 0.05	179.4 ± 14.0	> 0.05

group. The results were as shown in Table 4 and 5: (1) The GSH-Px activities in blood, heart, liver and muscle of the rats for KBD region group were lower than those of the rats for non-KBD region group and control group; (2) As compared with the control group, the SOD activities in blood and tissues of rats for non-KBD region group increased obviously and the SOD activity of the rats for KBD region group increased more obviously than that of the rats for non-KBD region; (3) The change in LPO content was not obvious. But the LPO contents in liver of the rats for KBD region group and non-KBD region group increased and had an obvious difference from those for the control group.

2. 30 Wistar rats with the same weight were divided into three groups: KBD region group, −OH blocked group and Se-supplement group. The abdominal cavity of the rats for three groups were injected with Tianshui FA, −OH-blocked FA of Tianshui and Tianshui

FA added with 0.1ppm Se, respectively, by taking a dose of 100 $\mu g\ g^{-1}$ body weight. At 72 hours after injecting, bloods in tail and heart, liver, gastrocnemius muscle were drawn and examined for the GSH-Px and SOD activities and LPO content of the rats. The −OH-blocked group and Se-supplement group were respectively compared with the KBD region group. The results were as shown in Table 6 and 7: (1) FA in KBD region with −OH groups blocked caused by FA in KBD region, without reduction in GSH-Px and SOD activities in blood and tissues and LPO content nearly disappeared; (2) The influence of Se-supplement on the effect of GSH-Px activity and LPO content of FA in KBD region FA was similar to that of −OH-blocked group, but Se-supplement had no effect on raising SOD activity caused by FA in KBD region.

DISCUSSION

The free radical theory is one of the theories of disease occurrence that develop fastest and have attracted more attention from scholars in recent years. The free radical initiators (mainly $\cdot O_2^-$ and $\cdot OH$, $^1O_2$ induced by $\cdot O_2^-$ and so on oxidize and attack abundant unsaturated fatty acid in cell membrane, cause lipid peroxidation, and produce lipid peroxide. Malondialdehyde (MDA) produced from the reactive products causes a series of reactions such as addition, scission, cross-linking with DNA, protein, enzyme and so on, and induce different pathological changes and diseases (Pryor, 1973; Leibovitz, 1980; Gross, 1987). The reaction caused by free radical is chain reaction. Therefore, cells in the tissues must eliminate timely extra free radical and lipid peroxide, and adjust and control the free radical reaction in normal physiological conditions to keep organism healthy. SOD and GSH-Px are two main kinds of antioxidase for adjusting and controlling free radical reaction in cells. The former is only a kind of enzyme known to eliminate $\cdot O_2^-$. The main role of latter is to eliminate peroxide inducing H_2O_2, (Leibovitz, 1980; Halliwell, 1986). Therefore, the pathologic process of KBD caused by the degradation products of organic matters (present in the grain and drinking water polluted) may be the process of peroxidation injury caused by lack of the ability to eliminate LPO mainly due to reduction in GSH-Px activity. Humic acids contain many stable free radicals. Organic matters have been proved to be the main source of inducing free radicals (Mason, 1982). The raising of SOD activity in blood and the tissues seen in this animal experiment shows that, under the action of the degradation products of organic matter, the cells in the tissues are always in the course of reaction leading to increase in free radical (cells in tibid had been confirmed by the ESR spectrum). Therefore, the action of free radical in the course of inducing pathological changes of KBD depends probably on the ability to improve adaptability of SOD activity and to eliminate extra free radical, and more attention should be paid to this regard.

As compared with HA, FA contains more oxygen and oxygen functional groups (Schinitzer, 1972). The injurious effect of FA on chondrocytes is always more than that of HA. Then we firstly thought of the pathological action of oxygen functional groups. And studies have confirmed that the injurious effect of active oxygen came mainly from hydroxyl free radical (·OH), (Halliwell, 1986; Schnitzer, 1965; Gross, 1987). Therefore, we compared the effects of organic acid reagents on chondrocytes and found that the injurious degree of cells was closely related to −OH groups contained in organic acid. Then, in the studies on animal experiment we have confirmed that the effects of humic acids in decreasing GSH-Px activity, raising SOD activity and increasing. LPO content may disappear after −OH groups being blocked by acetylation of humic acids. The above results make us sure that the peroxidation injury of tissues caused by degradation products of organic matter was induced by its oxygen functional groups (especially −OH group). As far the relation between oxygen functional groups and producing free radical, and whether oxygen functional groups provide source and good condition for producing of free radical will be further studied and confirmed.

To sum up, we believe that: 1. The cause of KBD is toxic degradation products of organic matter present in the grain and drinking water of KBD region; 2. The basic pathological process is peroxidation membrane injury induced by obviously decreasing of GSH-Px caused by oxygen functional groups in organic degradation products. Se deficiency provides good condition for the injury; and 3. The increasing of free radical reaction made the pathological process of KBD more serious. The degree of influence is related to the reactivity of SOD activity in cells membrane.

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