Studies on intestinal and muscle protein-bound carbohydrate components in experimental hypercholesterolemia—Effect of Annapavalasindhooram—An indigenous drug formulation

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Abstract

The changes in the protein-bound carbohydrate (PBC) components including glycosaminoglycans (GAGs) play an important role in atherosclerotic degeneration. In the present study, the variation in PBC and GAGs in serum and in tissues—small intestine and skeletal muscle of normal, atherosclerotic and Annapavalasindhooram (APS)-treated animals have been taken into consideration. The level of PBC components, mainly hexose, hexoseamine, sialic acid and hexuronic acid, generally increase in atherosclerotic condition. During atherosclerosis, a very mild increase in hyaluronic acid (HA) and heparan sulphate (HS) and a decrease in chondroitin sulphates A, C, B (C, A, C, C, C, B) and sulphated GAG/nonsulphated GAG ratio is observed. The treatment of hypercholesterolemic animals with APS brings some of the PBC components and GAG fraction to near normal. This leads to suggestion that APS may lower lipid level in atherosclerotic animals as reported earlier by altering PBC components which play an important role in the genesis of atherosclerosis.

Key words : Hypercholesterolemia, glycosaminoglycan, protein-bound carbohydrate, cholesterol lowering drug.

1. Introduction

Increasing incidence of cardiovascular diseases has led to the discovery of various hypo-lipidemic drugs which lower lipid level and induce regression of arterial lesion. But most of these drugs do not completely combat the disease. Some of them have harmful side effects also. In this connection, we have tried out Annapavalasindhooram (APS), a Sidha medicine formulated in our laboratory, which is established to have hypo-lipidemic character in the earlier experiments carried out in our laboratory[1].

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Annapavalasindhooram has Annabedi (Ferric sulphate) and "Pavalam" (*Corallium rubrum*) as the basic minerals. Coral reef and Annabedi are ground in the extracts or juices of seven herbs, each one having one or more desired properties such as cardiac tonic, liver stimulant, laxative, diuretic, expectorant and cholagoguic and calcined in seven stages by the method known as "Sputum" under the supervision of a qualified Sidha expert. The final residue is pulverised and this reddish brown fine powder (APS) is used for the investigations. It is a mineralo-herbal product with 16–18 % iron, 23–28% calcium, 3–4% magnesium and traces of manganese along with sulphate 15–20 % and phosphate 1–3%. It also contains 10–15% organic matter. Atomic absorption spectroscopic analysis revealed the presence of traces of copper and the total absence of zinc, mercury and lead.

In addition to lipid accumulation, atherosclerosis is associated with changes in the structural and functional integrity of the tissues affecting their strength, elasticity and flexibility. The integrity of the tissue structure depends on the molecular organization of its protein-bound carbohydrates mainly glycoproteins, mucoproteins and glycosaminoglycans. Abnormal level of these protein-bound carbohydrates has been suggested to play a very important role in the development of atherosclerosis[2,3].

Protein-bound carbohydrate formation is accelerated in tissues characterized by increased glycolysis[4]. Variation in the activities of glycolytic enzymes has been noted in intestine and skeletal muscle of atherosclerotic animals[5], and the changes in protein-bound carbohydrate components in these tissues in experimental hypercholesterolemia has been taken into consideration.

In the present study, the level of total protein-bound carbohydrate components has been investigated in small intestine and skeletal muscle of normal and hypercholesterolemic rats and under APS therapy. Glycosaminoglycanes (GAGs) were fractionated and alteration in each of these individual fractions during hypercholesterolemic condition and after APS therapy has also been studied.

2. Materials and methods

Albino rats derived from the Wistar strain obtained from the King's Institute of Preventive Medicine, Madras, used in the investigations, were fed goldmohur rat feed manufactured by M/s Hindustan Lever Ltd. and had access to food and water *ad libitum*. They were divided into two comparable groups, groups I and II. Group I animals were given the normal diet. Hypercholesterolemia was induced in group II animals by feeding an atherogenic diet which contained cholesterol 30·0%, coconut oil 1·5%, and thiouracil 25 mg/100 g body weight day as supplements to the commercial rat feed. The serum cholesterol was monitored periodically for all these animals. The animals were further divided into I *a*, I *b*, II *a* and II *b* at the end of 75 days, when the cholesterol-fed animals had serum cholesterol value around 250 mg/dl. Pilot experiments have shown that at this stage, there was positive atherosclerotic changes, such as

lipid deposition in the intimal and medial regions of the aorta. They were given the following dietary and therapeutic regimen, for the next one month, at the end of which period they were sacrificed and small intestine, skeletal muscle and serum samples were analysed.

Group I a — Continued on normal diet.

Group I b — Animals were on normal diet supplemented with 10 mg APS/day as a single dose for 30 days. This group served to assess toxicity, if any, by APS administration to rat.

Group II a — Continued on the atherogenic diet.

Group II b — Animals were continued on atherogenic diet and administered 10 mg of APS as a single dose for 30 days.

The 10 mg day dosage of APS was fixed on the basis of the dosages observed to be necessary to bring down serum cholesterol in rabbits, chicks and normal human volunteers studied earlier in the laboratory[6].

3. Biochemical investigation

Serum parameters : Apart from monitoring serum cholesterol[7] during the course of the feeding period, the animals were bled by heart puncture before sacrifice and serum was analysed for the following protein-bound polysaccharide components, total neutral hexoses[8], hexoseamine[9], fucose[10], sialic acid[11], nonamino polysaccharide (NAPS)[8], and hexuronic acid[12]. The values were expressed in mg/dl serum.

Tissue parameters : At the end of the experimental period, the animals were sacrificed immediately after blood collection by cervical dislocation and the whole of the small intestine and skeletal muscle samples from hind limb region were dissected out and chilled on ice. The intestine was washed by passing ice-cold saline to remove the intestinal contents. The aorta was dissected out and was used for histological studies.

The tissues were defatted with successive extractions at 60°C with ethanol and ether (3 : 1 v/v) and chloroform : methanol (1 : 1 v/v) twice with each solvent for 2 hr. The defatted tissue was subjected to papain digestion at 65–70° C in 0·1 M phosphate buffer pH 6·5 containing 0·005 M EDTA and 0·005 M cysteine hydrochloride according to the procedure of Scott[13] for 48 hr with the addition of free papain at the end of every 16 hr.

The protein-bound carbohydrate components such as total neutral hexoses, fucose, hexoseamine, sialic acid and nonamino polysaccharide were estimated in the papain extract using the procedures adopted for serum.

The tissue glycosaminoglycans (GAGs) were fractionated on cellulose column by the modified method of Prasannan and Kurup[14]. The concentration of glycosaminoglycans were expressed in μg of uronic acid/mg of dry defatted tissue.

4. Results

Figure 1 gives the cholesterol value of experimental animals at the end of 3 months. The normal animals have a cholesterol value of 80–90 mg/dl. At the end of the experimental period, group II a shows serum cholesterol value around 300 mg/dl, while group II b animals had a serum cholesterol level of 200 ± 4·2 mg/dl. The decrease in cholesterol level shows that the hypocholesterolemic effect of Annapavalasindhooram is significant. However, longer periods of drug therapy are to be investigated.

Complete regression of aortic lipid deposition was not observed in the APS-treated rats of group II b. However, the extent of lipid deposition was lesser than in group II a. The aorta of group I b appeared normal and were similar to group I a.

Table I gives the protein-bound polysaccharide components level in the sera of experimental animals. The level of hexose, hexoseamine, hexuronic acid, nonamino polysaccharide and sialic acid increases in hypercholesterolemic rats. After treatment with
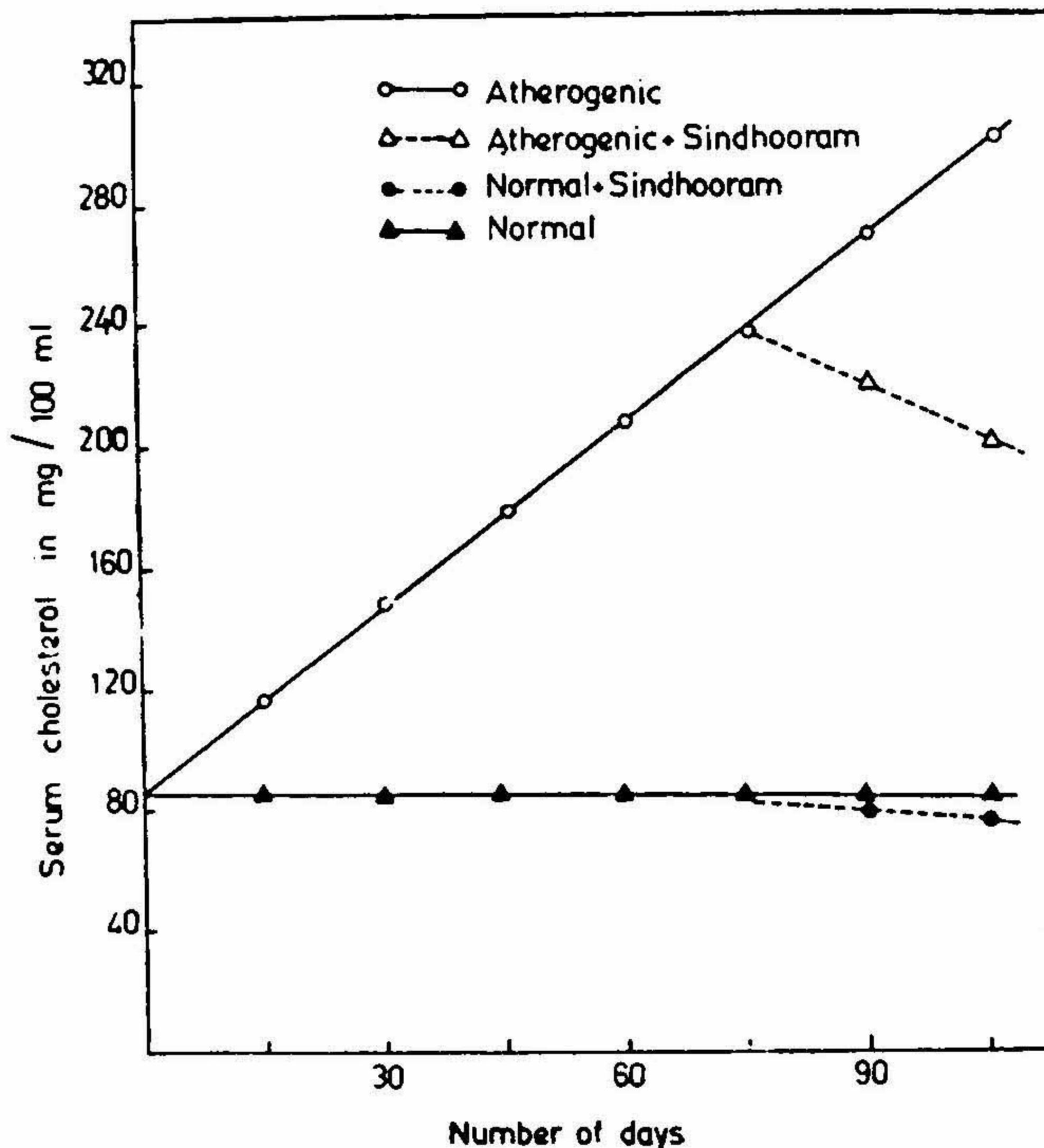

FIG. 1. Serum cholesterol levels in all the four groups of animals at the end of the experimental period.

Table I

Protein-bound carbohydrate components in the serum of experimental animals

Values are expressed in mg/dl of serum and are given as mean ± S.D. No. of animals in each group is given in parenthesis.

Protein-bound carbohydrate components	Normal Group I a (5)	Normal + APS Group I b† (5)	Atherogenic Group II a†† (5)	Atherogenic + APS Group II b††† (5)
Hexose	223·0±21·5	204·0±26·1	327·5±32·2**	251·0±21·3**
Hexoseamine	58·2±14·4	139·5± 3·5***	91·9± 7·8**	119·5±10·6*
Hexuronic acid	45·9± 3·4	36·5± 3·2**	72·8±14·1**	45·6± 3·8**
Nonamino polysaccharide	131·3±13·0	173·6± 7·5**	212·5±55·2*	181·1±25·4
Fucose	15·5± 2·5	13·3± 2·9	12·4± 5·1	15·1± 2·8
Sialic acid	89·7±32·9	206·7±25·6**	186·0±60·9*	151·8±43·7

† Group I b is compared with Group I a.   †† Group II a is compared with Group I a.   †††Groups II a and II b are compared.

The values are statistically significant at $p < 0.05$,   * $p < 0.05$,   ** $p < 0.01$,   *** $p < 0.001$.

Annapavalasindhooram, the levels of hexose and hexuronic acid come back to normal. The levels of sialic acid and nonamino polysaccharide decrease after treatment with Annapavalasindhooram (Group II b), though they do not come back to normal levels. Fucose level does not show much deviation from normal value in the cholesterol-fed animals of both groups II a and II b. However, an increase is observed in the hexoseamine, nonamino polysaccharide and sialic acid levels after treatment of normal animals with APS and also in APS-treated hypercholesterolemic rats.

Table II shows the protein-bound carbohydrate levels in the intestine and muscle of experimental animals. It is interesting to note that animals on atherogenic diet II a have elevated levels of hexose, hexoseamine and sialic acid in muscle and of hexose in intestine. Nonamino polysaccharide is lowered in muscle and intestine of hypercholesterolemic animals by about 50 %, while the lowering of fucose is less pronounced. Sialic acid is raised in muscle and lowered in intestine during hypercholesterolemic condition.

Administration of APS leads to a significant decrease in hexoses in the muscle and intestine of animals both on normal and atherogenic diet. Nonamino polysaccharides are lowered in APS-treated normal intestine (I b) and elevated in drug-treated hyperlipemic (II b) intestine, while they are lowered in both conditions in muscle. Sialic acid levels are increased by the administration of APS in muscle of animals on different diets.

Table II

Protein-bound carbohydrate components in the intestine and muscle of the experimental animals

Values are expressed in mg/gm of the dry defatted tissue and are given as mean ± S.D.   No. of animals in each group is given in parenthesis

Protein-bound carbohydrate components	Normal Group I a (5)	Normal + APS Group I b† (5)	Atherogenic Group II a†† (5)	Atherogenic + APS Group II b††† (5)
Intestine				
Hexose	14·5±3·4	4·0±1·8**	23·4±3·9*	7·3±1·4***
Hexoseamine	5·6±1·6	6·5±1·3	6·4±1·1	5·5±1·6
Nonamino polysaccharide	10·8±1·7	2·1±0·4***	3·6±0·6***	7·3±0·7***
Fucose	1·0±0·3	0·7±0·1*	0·7±0·1*	1·6±0·2***
Sialic acid	8·9±0·8	8·4±1·1	4·3±0·9***	8·4±1·6**
Muscle				
Hexose	5·8±0·6	1·6±0·7***	8·8±2·2*	5·1±0·9*
Hexoseamine	2·8±0·2	1·3±0·1***	3·5±0·3**	3·6±0·4
Nonamino polysaccharide	5·9±1·0	1·4±0·2***	2·7±0·2***	1·9±0·2**
Fucose	0·9±0·1	0·6±0·1**	0·7±0·1*	0·5±0·1
Sialic acid	1·5±0·3	2·7±0·3**	2·2±0·6*	3·0±0·6

† Group I b is compared with Group I a.   †† Group II a is compared with Group I a. ††† Group II a is compared with Group II b.

The values are statistically significant at $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

However, in the intestine, APS appears to have a protective effect in correcting the protein-bound sialic acid content in the hyperlipemic animals.

Analysis of glycosaminoglycan fractions in the intestine and muscle shows that the level of hyaluronic acid and heparan sulphate show an increase in muscle and not in the intestine of atherosclerotic animals, while chondroitin sulphates A, B, C decrease. The ratio of sulphated GAG to nonsulphated GAG decreases from $2·9 ± 0·3$ to $2·6 ± 0·2$ in the intestine and from $2·2 ± 0·3$ to $1·5 ± 0·1$ in the muscle of atherosclerotic animals. Annapavalasindhooram administration to hyperchclesterolemic animals brings the level of glycosaminoglycan fractions to normal in both the intestine and the muscle. APS administration to normal animals does not alter GAG levels in muscle and in intestine.

Table III

Glycosaminoglycan fractions in the intestine and muscle of the experimental animals

Values are expressed in µg of uronic acid/gm of the dry defatted tissue and are given as mean ± S.D. No. of animals in each group is given in parenthesis

Glycosaminoglycan fractions	Normal Group I a (5)	Normal + APS Group I b† (5)	Atherogenic Group II a†† (5)	Atherogenic + APS Group II b††† (5)
Intestine				
Hyaluronic acid	334·1±32·2	327·8±25·6	357·0±32·7	326·0±36·3
Heparan sulphate	405·8±58·2	391·4±40·0	450·6±76·3	398·4±79·0
Chondroitin sulphate A	154·6± 8·5	153·5± 4·9	127·0±21·2*	146·4± 8·3
Chondroitin sulphate C	232·9±62·3	220·2± 9·6	165·1±21·4	216·8±20·3*
Chondroitin sulphate B	179·4± 5·0	185·0± 4·8	137·6±21·8**	168·4±12·4*
$\frac{\text{Sulphated GAG}}{\text{Nonsulphated GAG}}$ ratio	2·9± 0·3	2·9± 0·4	2·6± 0·2	2·9± 0·2
Muscle				
Hyaluronic acid	244·6±50·8	239·7±47·7	331·9±24·5*	267·1±18·4**
Heparan sulphate	162·0±16·2	167·1± 9·6	222·7±20·1**	176·0±12·0**
Chondroitin sulphate A	111·5±10·6	121·4±10·6	80·5± 6·0**	106·5± 5·7***
Chondroitin sulphate C	133·6±11·3	137·1± 8·7	113·3± 7·2*	138·0± 3·7**
Chondroitin sulphate B	120·4±14·2	126·6± 6·7	91·4± 5·2**	120·2±10·5**
$\frac{\text{Sulphated GAG}}{\text{Nonsulphated GAG}}$ ratio	2·2± 0·3	2·4± 0·3	1·5± 0·1**	2·0± 0·1**

† Group I b is compared with Group I a. †† Group II a is compared with Group I a. ††† Group II a is compared with Group II b.

The values are statistically significant at $p < 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5. Discussion

The observation made on the serum cholesterol levels and the aortal histology indicates that in the short period of APS therapy, the lipid lowering effect is incomplete and that the lipid deposited in the aorta is not completely removed. However APS administration prevents further aortic lipid accumulation. Subsequent work in our laboratory[6] has shown that the changes in the aorta are reversed at a slow rate during APS administration, while the liver and intestinal metabolism respond early to APS administration,

The variation in the protein-bound carbohydrate components during hypercholesterolemic condition may arise due to a disturbance in the carbohydrate metabolism and its regulation. Mikhailova[16] has reported a retardation in the conversion of glucose to glycogen in atherosclerotic condition. In hypercholesterolemic rabbits, increased formation of glucose from glycerol due to enhanced activity of gluconeogenic enzymes has also been observed[5]. Moreover, the elevated fatty acid level inhibits glycolysis, which is accompanied by accumulation of glucose and glucose-6-phosphate[17]. Decreased activity of phosphofructokinase[18], aldolase and lactate dehydrogenase[5] has been proved in experimental atherosclerosis, suggesting the inability of atherosclerotic tissue to utilise glucose properly *via* glycolysis. Thus the accumulated glucose, mainly as its phosphoester in the cell, is known to enter alternate pathways such as sorbitol pathway[19], protein-bound carbohydrate synthesis[20] leading to basement membrane thickening. The increased availability of hexose, hexoseamine, hexuronic acid and sialic acid in serum and tissues as seen in our experiments may be the triggering factor for increased glycoprotein synthesis and its accumulation in the basement membrane.

Changes taking place in the glucose catabolism in hyper-lipaemic condition favour the stimulation of minor pathways producing enhanced levels of hexose, hexoseamine and hexuronic acid, as the uridine diphosphate (UDP) derivatives. The sugars, amino sugars and uronic acid may be incorporated into the cell membrane providing increased polar surfaces, thereby further modifying the permeability of the membrane to lipid components, which is the first step in lipid deposition and development of atherosclerotic plaques[21-22].

The treatment of hypercholesterolemic animals with the drug (APS) brings hexose, hexuronic acid level to near normal in serum and lowers hexose in intestine and muscle. This suggests that Annapavalasindhooram may lower lipid level by possible alteration of a few of protein-bound carbohydrate components, which regulate the permeability of the membrane to lipids.

It may be noted that intestinal content of nonamino polysaccharide and sialic acid are greatly reduced in hypercholesterolemic animals while their serum levels are elevated. The increases in the serum level of these components may be in part contributed by the intestinal depletion of these compounds. APS administration lowers serum nonamino polysaccharide and sialic acid, while reverse is observed in intestine, possibly due to the regeneration of the tissue.

The ratio of nonamino polysaccharide levels in serum : muscle : intestine are 3:3:4 in normal animals and 6:1·5:2 during hypercholesterolemic condition. This suggests that the increase in nonaminopolysaccharide level in serum is compensated by a proportional decrease in intestine and muscle. APS treatment partially corrects the altered nonamino polysaccharide levels in hypercholesterolemic animals.

Treatment of normal animals with APS lowers hexose, fucose and nonamino polysaccharide in intestine and hexose, hexoseamine, nonamino polysaccharide and fucose

in muscle. Previous studies in our laboratory have shown that treatment of normal animals with the drug lowers lipid level in liver, kidney and intestine[1]. Moreover, our studies have shown that treatment of normal animals with APS lowers serum cholesterol level marginally. This suggests that APS may lower lipid level in normal animals by interfering with protein-bound carbohydrate metabolism. Conclusions can be arrived at on the action of APS on normal cells, only after elaborate studies on the synthesis of these components.

The moderate increase in the level of hyaluronic acid and heparan sulphate during hyperlipemia may be due to increased synthesis of hexoses, N-acetylated hexoseamine and hexuronic acid. The decrease in the level of chondroitin sulphates A, B and O in group II *a* animals is likely to be due to impaired sulfation or due to increased activity of their degrading enzymes. Similar changes have been reported by Silberberg *et al* in guinea pigs[13] and Peters *et al* in rabbits[24]. The decreased ratio of sulphated GAG to nonsulphated GAG in hypercholesterolemic rats suggests a minimised rate of formation of sulphated GAG, as suggested earlier by Vijayakumar and Kurup[26]. Treatment of hypercholesterolemic animals with APS corrects these imbalances showing that apart from its plasma cholesterol lowering effect, prevention of lipid deposition, APS favours the sulphation of GAG, an essential feature in the correction of atherosclerosis.

Annapavalasindhooram treatment to atherosclerotic animals restores some of the protein-bound carbohydrate components to near normal values suggesting that APS has a role to play in secondary metabolite formation and degradation.

Acknowledgements

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