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Evaluation of Snail Mucin Motifs as Rectal Absorption Enhancer for Insulin in Non-Diabetic Rat Models

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The use of snail mucin motifs as rectal absorption enhancer for insulin has been evaluated. The mucin motifs were extracted from the giant African snail *Archachatina marginata* by differential precipitation with acetone. The mucin motifs were found to have a molecular weight of 5780 Da and an isoelectric point of 3.4. At the concentrations evaluated, the mucin exhibited rectal absorption enhancing property for the administration of insulin in rats. The % basal blood glucose level of the rats that received the batch of suppositories containing no mucin were consistently above 100% except at the ninetieth minute when it came down slightly to 97.2%. Rats dosed with the batch containing 7%w/w suppositories showed the greatest blood glucose reduction with mean % basal blood glucose concentration of 61.2%. Batches of the suppository containing 5% and 7% mucin showed more marked and consistent lowering in blood glucose concentration than the other batches containing lower amounts of the rectal absorption enhancer. The batch with 7% mucin reduced the basal glucose level to 44% within 2 h of administration of the glycero-gelatin suppository loaded mucin.

Key words evaluation; snail mucin motif; rectal absorption enhancer; insulin

It was once believed that rectal administration of a drug provided a means of avoiding degradation by the liver and damage of the liver by the drug. Recent investigations have shown that avoiding the first passage through the liver is possible. The extent of this effect cannot be generalized since it will depend on the actual part of the rectum through which the drug is absorbed.[1] Thus keeping the drug in the lower part of the rectum is advisable.

Mucin or mucus glycoproteins are a family of polydisperse molecules designed to carry out multiple tasks at mucosal surfaces throughout the body.[2] Mucins are high molecular weight epithelial glycoproteins with a high content of clustered oligosaccharides *O*-glycosidically linked to tandem repeat peptides rich in threonine, serine and proline.[3,4] They are rich in cysteine residues involved in sub unit crosslinking that form a macromolecular complex.[5] They contribute to the mucus gel barrier and are part of the dynamic, interactive, mucosal defensive system. Over the years, several studies carried out on mucus glycoprotein from many organs have suggested that these macromolecules consist of subunits held together by interchain disulphide bonds and further stabilized by non-covalent interactions.[6]

Studies have shown that immobilization of thiol groups to well-established mucoadhesice polymers resulted in thiolated polymers, which can form disulphide bonds with cysteine-rich sub-domains of mucus proteins.[7] These polymers were also shown to exhibit strong permeation penetrating properties.[8] Mucin administered exogenously formed disulphide linkages between its cysteine domain and those of the endogenous mucin increasing the gel network and hence the viscosity of the mucus. Mucins can, therefore, be harnessed as absorption modifier as they will increase the contact time of co-administered drugs and possibly serve as sustained release polymers. In this study snail mucin is evaluated as a rectal absorption enhancer for insulin. The mucin from this source has earlier been evaluated for toxicity and found to be safe when used *via* the nonparenteral route.[9]

MATERIALS AND METHODS

Materials The following chemicals were used without further purification: soluble insulin 40 U/ml (Knoll, China), gelatin (Merck, Germany), glycerol (Merck, Germany), distilled water prepared from an all-steel still (Kottermann, England), phenobarbitone sodium 200 mg/ml (Renaudin, France)

Animals Mature male Wistar albino rats weighing between 250 and 300 g were obtained from the Department of Veterinary Medicine, University of Nigeria and fed on 'chicks marsh' (Top Feeds, Nigeria) were used for the study. After the purchase, all the rats were allowed to equilibrate in standard and conditioned animal houses at the Department of Food Science and Technology, University of Nigeria, for a period of 7 d before use.

Snails Fresh giant African land snails, *Archachatina marginata* (Fam. Arionidae) were bought from Nsukka central market. The extraction of mucin was done in our laboratory following standard procedures.

Methods. Preparation of Mucin After procurement, the shells of the snails were cracked open and a spirally coiled rod inserted to remove the fleshy body from where the excretory materials were removed. The fleshy parts were then placed in 250 ml of water and washed until the mucin was completely washed off. These washings were pooled together in a plastic bucket, precipitated using chilled acetone and lyophilized in a lyophilizer. The grey-brown lyophilized flakes of the snail mucin were pulverized into fine powder using an end runner mill and stored in an airtight container until used.

Molecular Weight Determination by Gel Permeation Chromatography The Sephadex G-100 was allowed to swell in excess buffer (0.05 M Tris buffer, pH 7.5) for the recommended time of 3 d at room temperature, in order to obtain satisfactory flow rates through the gel and thus good separation. The column was poured and equilibrated with the buffer. The void volume was established with blue dextran (V_o) (5 mg/ml; weight average-molecular weight 2×10^6; read at 625 mμ. The sample (snail mucin dispersion) was applied.

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The volume at which the snail mucin eluted from the column was determined (V_e). Four standards (10 mg/ml) were applied to the column in runs of 2 standards per run to determine the elution volumes (V_e) of the standards. The standards used were methyl red, bovine serum albumin (BSA), ribonuclease and ovalbumin. The K_{av} for the test fraction (snail mucin) and the standards were calculated using Eq 1:

$$K_{av}=\frac{V_e-V_o}{V_t-V_o} \quad (1)$$

where V_e is elution volume of the (active) material, V_o is elution volume of blue dextran, V_t is the total volume of gel bed ($\cong \pi r^2 h$, r is radius of the column and h is height of the column).

To prepare the standard curve, K_{av} of the standards was plotted against log. molecular weight. From the K_{av} of the test material (snail mucin), which was unknown, the molecular weight was determined from the standard curve.

Determination of Snail Mucin Isoelectric Point Seven buffer solutions of different pH values ranging over 3.2—5.7 were made in 7 test tubes. A 0.5 ml volume of 2% snail mucin (protein solution) was added to each test tube and the contents mixed. The test tubes were shaken and noted for the appearance of a cloudy solution. A 2 ml quantity of ethanol was added to each of these test tubes and visual estimation of the solution degree of clouding was determined. At this pH, the electrophoretic mobility is zero because Z in the equation below is zero:

$$v=E_i Z/f \quad (2)$$

Where: v is velocity of migration of a protein in an electric field; E_i is the strength of the electric field; Z is the net charge on the protein while f is the frictional resistance, which is a function of size and shape.

Preparation of Insulin Suppository Five batches of insulin glycero-gelatin suppositories were produced each containing varying quantities of mucin. Calculations were made for thirty suppositories per batch.

A 5.04 g quantity of gelatin was weighed out with an electronic balance (Ohaus, China) and dissolved in 8 ml of water and heated to boiling. Glycerol, previously heated to 100 °C, was added to the gelatin paste and the mixture was heated on a water bath with gentle stirring until solution was complete. The mixture was removed from heat and stirred gently until its temperature came down to 30 °C. A 3.75 ml aliquot of insulin injection (equivalent to 150-U) was incorporated into it. The product was poured into 1 g steel moulds already lubricated with liquid paraffin and quickly transferred to an ice bath and allowed to set. For the rest of the batch, after production and cooling of the glycero-gelatin base to 30 °C, the appropriate amounts of mucin—0.6 g, 1.2 g, 1.8 g and 2.4 g were weighed out and incorporated into batches B, C, D and E respectively. Each batch contained 3.75 ml of insulin incorporated into the mixture in a similar manner as the batch A preparation. The molds were transferred to an ice bath and the suppositories were allowed to set and stored in well-closed containers under refrigeration.

Physical Tests. Weight Uniformity Twenty suppositories were drawn randomly from each batch and weighed together using an electronic balance (Ohaus, China). Their average weight was determined. They were then weighed singly and the deviations from the mean were calculated.

Disintegration Time Five suppositories were drawn randomly from each batch. They were placed in a disintegration test unit (Erweka,) and the mean time taken for them to disintegrate in distilled water medium and pass through the screen was recorded.

Resistance to Rupture Five suppositories were randomly drawn from each batch and were subjected to the European Pharmacopoeia test for resistance to rupture using modified equipment.

Rectal Absorption Studies The male Wistar rats aged 3 months and weighing between 200—250 g, were randomly divided into 5 groups of 4 rats per group. They were fasted for 18 h with free access to water. The rats were anaesthetized using phenobarbitone sodium (50 mg/ml administered as 0.1 ml/kg body weight). Under full anaesthesia the basal blood glucose concentration was taken using a glucometer (Accu-chek®, Roche, Switzerland) by the tail snipping method. A quantity of the suppository equivalent to 5-U of insulin/kg body weight of the rats was inserted into their rectum. Blood samples were taken from the tail vein at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 6 h and examined for blood glucose concentration. The post dose levels of the blood glucose were expressed as a percentage of the predose level. The percent basal blood glucose concentration was plotted against time for the various groups.

Pharmacodynamic Analysis This was done following an earlier method using the trapezoid rule.[10] Parameters determined were *AAC* (area above the serum glucose levels *vs.* time curve), time taken to achieve minimum glucose concentration (T_{min}) and the minimum glucose level attained (GL_{min}), as well as the percent maximum lowering of glucose at T_{min}.

Statistical Analysis The ANOVA test was applied to statistically analyse the results. Values ≤ 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Some Physicochemical Properties of the Mucin The molecular weight of the snail mucin motifs was estimated from a calibration curve of standards proteins to be 5780 Da. This value is within the useful working range of Sephadex G-100, which depends on the extent to which the gel has swollen, and evidently varies also from lot to lot.[11,12] Molecular weights of polymers affect their behaviour when used as mucoadhesive agents especially in contact with mucin. Investigation on the effect of molecular weight of 4 viscosity grades of sodium carboxymethylcellulose (SCMC) by Smart *et al.* [13] showed that molecular weight affects bioadhesion and that optimum is 8600 Da Polymers with molecular weights greater than 100000 have been reported to exhibit maximum adhesion.[14] This was also determined by their molecular weights.[15] Park and Robinson[16] studied the binding of various polymers to the mucin and epithelial cell surface and noted the importance of ionizable group. They found that polymers with ionizable groups were generally most adhesive.

The isoelectric point of the snail mucin was found to be 3.4. The Isoelectric point is the pH at which there is no net electric charge on a protein. Below the isoelectric point pH,

the snail mucin is positively charged and above, it is negatively charged.

Considering the fact that mucus is negatively charged, any mucoadhesive material with a net positive charge *e.g.* gelatin will produce a relatively high degree of bioadhesion of longer duration, while a mucoadhesive polymer such as carboxymethylcellulose (CMC) with a net negative charge will produce a relatively low degree and duration of bioadhesion.[17] Different parts of the body secret mucus with different degrees of negativity.

Physical Properties of the Formulated Suppositories The result of the weight uniformity of the suppositories shows that the suppositories exhibited high degree of weight uniformity. There were minimal intra- and inter-batch variations. The weights ranged from 1.45±0.01 to 1.50±0.01 g for the suppositories containing mucin while a higher variation from these figures was noted for the suppository batch without mucin. However, intra batch variation was minimal as the standard deviations were very low. This is expected of suppositories produced using molds where careful pouring and cooling has been maintained.

The disintegration time studies carried out on the various suppository batches showed that the batch without any mucin, batch A (control) disintegrated within the shortest time (16.3±0.07 min) while batch E (containing 7%w/w mucin) took the longest time (25.5±0.08 min) to disintegrate. Generally the disintegration time for all the batches of the suppositories containing mucin ranged from 19.7±0.3 to 25.5±0.08. It should be noted that the disintegration times were long because most suppositories did not disintegrate; they rather eroded slowly. It seems that the presence of mucin increased the viscosity of the glycerogelatin materials probably due to cross-linking with the thiol groups in gelatin[8] resulting in formation of a gel matrix that impeded the movement of water into the suppository mass.

The results of the studies on resistance to rupture carried out on the suppositories show that batch A suppositories (without mucin) had the lowest resistance to rupture. They ruptured under an average force of 3.1 N. Resistance to rupture of the suppositories increased with increase in concentration of mucin except that the inter batch variations were narrow. For the batch containing 2% w/w muicn, the resistance to rupture was 4.2±0.1 N while those for the batches containing 3%w/w and 5%w/w were 5±0.1 N and 5.1 N respectively. The differences were not statistically significant ($p \leq 0.05$). The difference in resistance to rupture between the suppositories containing the least amount of mucin (4.2±0.1 N) and that containing the highest amount (6.1 N) was however, significant ($p \leq 0.05$). The resistance to rupture also increased with increasing concentration of mucin. Mucin probably increased the elasticity of the suppositories thereby increasing their resistance to permanent deformation. Batch A suppositories containing no mucin had the lowest resistance to rupture.

Figure 1 shows the % basal blood glucose concentration *vs.* time curves. Animals dosed with the suppositories showed a decrease in % basal blood glucose concentration within the first hour. However, the batch with the highest amount of mucin showed the highest pharmacodynamic effect.

Animals in all the groups that received suppositories containing mucin showed a decrease in % basal blood glucose concentration within the first 30 min. The action of mucin as a rectal absorption enhancer may be related to its affinity to biological surfaces. Being of animal origin, it may possess greater biocompatibility than non-biopolymers. It is simply mucin–mucin interaction; that is, the snail mucin interacting with the intestinal mucin. The molecular bridges which result from polymer self-diffusion account for the adhesive strength. It has been stated that mucus turn over is what affects rate of drug absorption rather that just adhesion.[18] The presence of additional mucin will enhance the mucus thickness on the mucosal surface of the intestinal epithelium. Thus the greater the mucosal layer the greater the amount of insulin absorbed. Apart from mucus thickness the property of the adhesive materials is also important. Mucus gel is a thick secretion composed mainly of water, electrolyte and a mixture of several glycoproteins, which themselves are composed of large polysaccharides.[19] This mucus gel is held together by either primary disulfide bonds or secondary bonds (electrostatic and hydrophobic interactions).[13] These cohesive mucin–mucin forces are the rate-limiting step in bioadhesion of several polymers.[20] Therefore, the bioadhesive bond depends on the strength of the mechanical bonds within the mucus.

Table 1 shows the pharmacodynamic parameters calculated. The area above the serum glucose levels *vs.* time curve was proportional to the amount of muicn in the system. When the mucin concentration was increased from 2 to 7 %w/w the *AAC* was doubled. However, the time for peak concentration increased. This shows that increased mucin concentration does not increase the rate of absorption but the

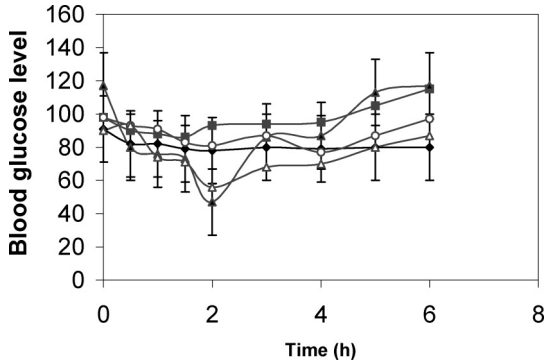

Fig. 1. Effect of Mucin on Blood Glucose Levels *vs.* Time Profiles after Administration of Glycero-Gelatin Suppositories to Rats

Each point is representative of an average of 4 readings; mucin concentration: ■ 0%, ○ 2%, ◆ 3%, △ 5%, ▲ 7%.

Table 1. Various Pharmacodynamic Parameters Calculated for the Glycero-Gelatin Suppositories Containing Various Proportions of Mucin

Mucin conc. (%w/w)	*AAC* (%h)	T_{min} (h)	GL_{min} (%)	$GLow_{max}$ (%)
0	60.54±10.23	1.90	96.00	4.00
2	80.70±3.55	1.90	84.60	15.40
3	88.25±10.48	1.90	76.75	23.25
5	140.53±2.68	2.00	55.25	44.75
7	160.28±5.45	2.00	44.00	56.00

AAC is area above the serum glucose levels *vs.* time curve. T_{min} is time taken to achieve minimum glucose concentration. GL_{min} is the minimum glucose level attained. $GLow_{max}$ is maximum lowering attained at time T_{min}.

total amount absorbed is increased. This is because mucin, as earlier mentioned, forms disulphide bonds increasing the viscosity of the rectal mucous layer. The minimum blood glucose level attained was also highest for the mucin suppositories containing 7%w/w mucin. When no mucin was incorporated, the minimum blood glucose attained was negligible (only 96%). The maximum level of blood glucose lowering ($GLow_{max}$) for this batch of suppositories was only 4% while for the batches containing mucin the $GLow_{max}$ ranged from 15 to 56%.

The higher amount of blood glucose reduction produced by batches D and E suppositories containing 5%w/w and 7% of mucin as compared to the control might be attributed to this effect as well as the release of insulin at the periphery of the suppositories before the activation of mucin. Rats that received batch B suppositories (containing 2%w/w mucin) showed faster recovery than those containing higher amount. The concentration of mucin in this batch probably was not enough to impart sustained/prolonged release property to the dosage form. Although batch D suppositories (5%w/w mucin) showed high reduction in blood glucose concentration, batch E suppositories (7%w/w mucin) performed better since it gave a more uniform rate of blood glucose lowering.

From the analysis of the results, it was found that the differences in the effect of the various concentrations of mucin on the absorption of insulin were not significant. Statistically, only the batch containing 5 and 7% mucin showed significant differences ($p>0.05$).

CONCLUSION

The use of mucin motifs as rectal absorption enhancer is presented in this work. The mucin motifs were effective as ehancer at concentrations of 5 and 7%w/w in glycerol-gelatin suppository as indicated by the plasma glucose *vs.* time profiles.

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