A study of the anti-diabetic agents of camel milk

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Abstract. The number of people diagnosed with type 2 diabetes has risen steeply recently exhausting the ability of health care systems to deal with the epidemic. Seventy-five percent of people with diabetes live in low- and middle-income countries. The largest populations of diabetics are in China and India, with many of those people living in extreme poverty. Combined forces of governmental health care, charities and donation of pharmaceutical companies would not be able to cope with the financial demands needed for medicaments and treatments for these people. Therefore, it is worth looking into traditional folk remedies to find if there is any scientific merit to justify their claims for alleviating symptoms of diabetes. There is a traditional belief in the Middle East that regular consumption of camel milk helps in the prevention and control of diabetes. Recently, it has been reported that camel milk can have such properties. Literature review suggests the following possibilities: i) insulin in camel milk possesses special properties that makes absorption into circulation easier than insulin from other sources or cause resistance to proteolysis; ii) camel insulin is encapsulated in nanoparticles (lipid vesicles) that make possible its passage through the stomach and entry into the circulation; iii) some other elements of camel milk make it anti-diabetic. Sequence of camel insulin and its predicted digestion pattern do not suggest differentiability to overcome the mucosal barriers before been degraded and reaching the blood stream. However, we cannot exclude the possibility that insulin in camel milk is present in nanoparticles capable of transporting this hormone into the bloodstream. Although, much more probable is that camel milk contains 'insulin-like' small molecule substances that mimic insulin interaction with its receptor.

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Introduction

Mature insulin is a protein of 51 residues (21 in A chain and 30 in B chain) produced in specialized beta cell islet of the Langerhans in the pancreas. Insulin binds on transmembrane tyrosine kinase receptor (insulin receptor) present in liver, muscle and cells in the fat tissues and stimulates increase glucose uptake from blood and converts it into glycogen to store in the liver and muscles. Insulin regulates carbohydrate and fat metabolism in the body. Failure to control insulin level leads to diabetes mellitus type 1 or 2. Patients with type 1 and ~40% of type 2 diabetic patients need insulin to control their blood glucose level. Type 2 diabetes is the most common and results from insulin resistance, a condition in which cells fail to use insulin properly.

The number of people diagnosed with type 2 diabetes has risen steeply in last decades severely exhausting the ability of health care systems to deal with the epidemic. Over 300 million people worldwide have diabetes and this most likely will rise to 500 million within the next 20 years. Seventy-five percent of people with diabetes live in low- and middle-income countries and according to prognostics Africa will experience a largest increase in the next generation. The highest incidence of this disease is in the Arabic Middle East, but the largest populations of diabetics are in China and India, with many of those people living in extreme poverty (1-5). According to a 2005 World Bank estimate, >40% of the total Indian population falls below the international poverty line defined as an income less than US$1.25 a day (Wikipedia, 2011). Combined forces of governmental health care, charities, and donation of pharmaceutical companies would not be able to cope with the financial demands needed for medicaments and treatments for these people. Therefore it is worth looking into traditional folk remedies to investigate if there is any scientific merit to justify their claims for alleviating symptoms of diabetes.

The traditional belief in the Middle East is that regular consumption of camel milk helps in prevention and control of diabetes, it has also been reported that camel milk can have such properties (6-8). This is a tempting hypothesis since over a few generations the Arab population has drastically changed its diet including drastic reduction of camel milk consumption. This was accompanied by a robust rise of incidence of diabetes. Two

independent groups studied influence of regular consumption of camel milk on diabetes and have reported a substantial reduction in the mean dose of insulin needed to obtain glycemic control (6,8,9) and improvement of fasting blood sugar (227.2±17.7 vs. 98.9±16.2 mg/dl), HbA1c (glucosylated hemoglobin) (9.59±2.05% vs. 7.16±1.84%), serum anti-insulin antibodies (26.20±7.69 vs. 20.92±5.45 μU/ml), urinary albumin excretion (25.17±5.43 vs. 14.54±5.62 mg/dl/24 h), reduction of daily insulin dose (48.1±6.95 vs. 23±4.05 units), and body mass index (18.43±3.59 vs. 24.3±2.95 kg/m^2) in randomized human study (9). No mechanism was provided to explain this phenomenon.

In different studies it was found that regular consumption of camel milk for a few months significantly improved the condition of diabetic patients and experimental animals (6-8,10). Zero prevalence of diabetes in camel milk drinking population and the results of use of camel milk in controlled clinical trials on diabetic humans and animals are highly encouraging to use it as natural therapy for the prevention and treatment of diabetes (6-8,10,11). Such beneficial effects of camel milk might be due to presence of insulin in the milk or some other substance(s) able to modulate glucose level. It contains higher level of insulin than milk from other animals (12) but to be effective it would have to be absorbed directly in the buccal cavity or completely proteolytically protected during passage through stomach and absorbed in the intestine. Camel milk is unique in the sense that it does not respond to acidic agents like other animal milk, possesses different casein content and much larger lipid micelles (13).

Literature review suggests following possibilities: i) insulin in camel milk possesses special properties that make absorption into circulation easier than insulin from other sources or cause resistance to proteolysis; ii) camel insulin is encapsulated in nanoparticles (lipid vesicles) that make possible its passage through stomach and entry into circulation; iii) some other elements of camel milk make it anti-diabetic.

In this study we are trying to understand the role of insulin in camel milk using bioinformatic tools. Sequence, structure similarity and literature review suggest that camel insulin similar to water buffalo and bovine does not possess any properties that should make it more resistant to proteolysis and easier to be absorbed into the circulation. There is no evidence that cow milk has any anti-diabetic properties albeit it does include insulin at lower level (12). However, it cannot be excluded that insulin if encapsulated in nanoparticles can cross digestive track walls. Lastly it is possible also that camel milk contains unidentified small molecules of 'insulin-like' regulatory value or of protease inhibitory properties to prevent proteolysis.

Materials and methods

Insulin sequences from different organisms were obtained from UniProt web search engine (http://www.uniprot.org/). Camel insulin (UniProt id: P01320) was used as a template for sequence in PSI-BLAST. The homologous insulin sequences from animals and plants were selected and subjected to multiple sequence alignment performed by Jalview (http://www.jalview.org/). The Multiple Sequence Alignment was color coded according to conservancy. The amino acid sequences of insulin were used to construct phylogenetic tree using BLOSUM62 from MAFFT Multiple Sequence Alignment (http://www.jalview.org/). The alignment quality of the amino acid sequences is based on BLOSUM62. Conservation among insulin sequences were calculated according to Livingstone and Barton. After multiple sequence alignment, consensus sequence represents the most common residues at a particular position. Quality measures the inverse likelihood of unfavorable mutations in the multiple aligned insulin sequences.

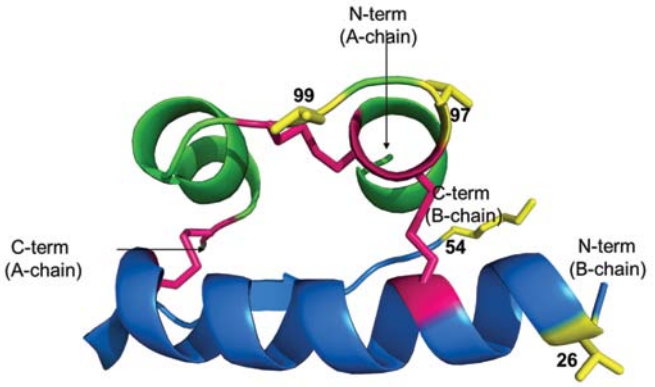

Figure 1. 3D structure of human insulin (1XDA). A-chain (green) covalently connected via disulfide bonds (magenta) to B-Chain (blue) of insulin. Camel insulin differs from human insulin at four positions (Val26Ala, Thr54Ala, Thr97Ala, Ile99Val).

Digestive pattern of different insulin was performed by online software, peptide cutter (http://web.expasy.org/peptide_cutter/). The number of the cut sites for pepsin at pH 1.3 and 2.0, trypsin and chymotrypsin with high and low specificity were recorded.

Protein structure modeling. An internet service I-TASSER server was used for protein structure and function predictions. It allows to automatically generate high-quality predictions of 3D structure based on amino acids sequence (14,15).

Results and Discussion

Proteolysis sites of digestive proteases in different types of insulin of different species. Models of human and camel insulin are essentially the same as predicted by I-TASSER (Fig. 1) (14,15). We hypothesized that camel insulin is protected from digestive enzymes in the stomach and thus absorbed in the intestine. The numbers of calculated cut sites for different types of insulin were the same for camel, human, bovine, goat, buffalo, sheep and pig insulin (Table I). The preferred cut sites for pepsin are Phe, Tyr, Trp and Leu. Trypsin prefers Arg and Lys at P1 while chymotrypsin preferentially cleaves at Trp, Tyr and Phe in position P1 (high specificity) and to a lesser extent at Leu, Met and His (low specificity). Camel insulin differs from human insulin by four mutations and from bovine and buffalo by just one mutation. None of the mutations affect specificity toward digestive enzymes. Therefore, camel insulin should be identical to human, bovine, buffalo, goat, sheep and pig insulin in terms of susceptibility toward proteolysis. Thus, when camel insulin comes in contact with the proteases of digestive track it should be digested like other mammalian insulin unless otherwise protected.

Table I. Proteolysis sites of digestive proteases in different types of insulin.

Insulin	Uniport accession no.	No. of cleavages				
		Pepsin pH 1.3	Pepsin pH >2.0	Trypsin	Chymotrypsin high specificity	Chymotrypsin low specificity
Human *(Homo sapiens)*	P01308	22	15	2	7	15
Camel *(Camelus dromedaries)*	P01320	22	15	2	7	15
Bovine *(Bos taurus)*	P01317	22	15	2	7	15
Water buffalo *(Bubalus bubalis)*	Q25C78	22	15	2	7	15
Domestic goat *(Capra hircus)*	P01319	22	15	2	7	15
Elephant *(Elephas maximus)*	P01318	22	15	2	7	15
Sheep *(Ovis aries)*	P01316	22	15	2	7	15
Whale *(Physeter macrocephalus)*	P67974	22	15	2	7	15
Chimpanzee *(Pan troglodytes)*	P30410	22	15	2	7	15
Hamster *(Cricetidae sp.)*	Q7M0G1	22	15	2	7	15
Pig *(Sus scrofa)*	P01315	22	15	2	7	15
Rabbit *(Oryctolagus cuniculus)*	P01311	22	15	2	7	15
Dog *(Canis familiaris)*	P01321	22	15	2	7	15
Cat *(Felis catus)*	P06306	22	15	2	7	16
Horse *(Equus caballus)*	P01310	22	15	2	7	15
Muscovy duck *(Cairina moschata)*	P68243	21	14	2	6	14
Goose *(Anser)*	P68245	21	14	2	6	14
Turtle *(Trachemys scripta)*	P69048	21	14	2	6	15
Ostrich *(Struthio camelus)*	P67969	21	14	2	6	15
Turkey *(Meleagris gallopavo)*	P67968	21	14	2	6	15
Alligator *(Alligator mississippiensis)*	P12703	20	13	3	6	14
Opossum *(Didelphis marsupialis virginiana)*	P18109	22	15	2	6	15
Chinchilla *(Chinchilla)*	Q5BVF6	21	14	2	7	16
Viscacha *(Lagidium viscacia)*	Q5BVF4	18	11	3	6	15
Mouse *(Mus musculus)*	E0CXX7	21	14	2	7	16
Bat *(Rhinolophus ferrumequinum)*	B2KIN7	22	15	2	7	15
Crab-eating macaque *(Macaca fascicularis)*	P30406	22	15	2	7	15
Guinea pig *(Cavia porcellus)*	P01329	15	10	3	6	12
Jack-bean *(Canavalia ensiformis)*	Q7M217	22	15	2	7	15
Camel's foot tree *(Bauhinia purpurea)*	721138A	22	15	2	7	15
Cowpea *(Vigna unguiculata)*	P83770	22	15	2	7	15

Insulin sequences. It is commonly believed that insulin sequences of different types of species are highly conserved (16-18). However, as shown in Figs. 2 and 3 some species may differ from human by as many as 18 amino acids (out of 51) in mature form of insulin. Camel insulin is identical to bovine and water buffalo, varying from human in Thr54Ala, Thr97Ala, Ile99Val. There is a contradiction on additional variation in camel insulin sequence at Val26Ala as reported byUniProt (19), while Al-Swailem *et al* reports only Thr54Ala, Thr97Ala, Ile99Val (20).

According to early studies by Pullen *et al* a number of conserved surface residues, forming the 'classical binding surface', were most likely involved in the insulin receptor binding (Gly90, Gln95, Tyr108, Asn110, Val37, Tyr41, Gly48, Phe49, Phe50, Tyr51) (21). The subset of this binding surface (Asn110, Phe49, Phe50, Tyr51), was later proposed to be essential for negative cooperativityin receptor binding by De Meyts *et al* (22) and confirmed by Xu *et al* (23). Two insulin mutations known to cause insulinopathy resulting in mild symptoms similar to diabetes type 2 are in this region (insulin Los Angeles: Phe-49-Ser) (24), (insulin Chicago: Phe-50-Leu) (25), see Fig. 4. Although insulin is such a small protein itself it forms dimers that further associate into hexamers important for this enzyme stability. The other residues such as Leu42 and Leu102 that are involved in hexamer-forming surfaces, are engaged also in receptor binding (26). In addition to the

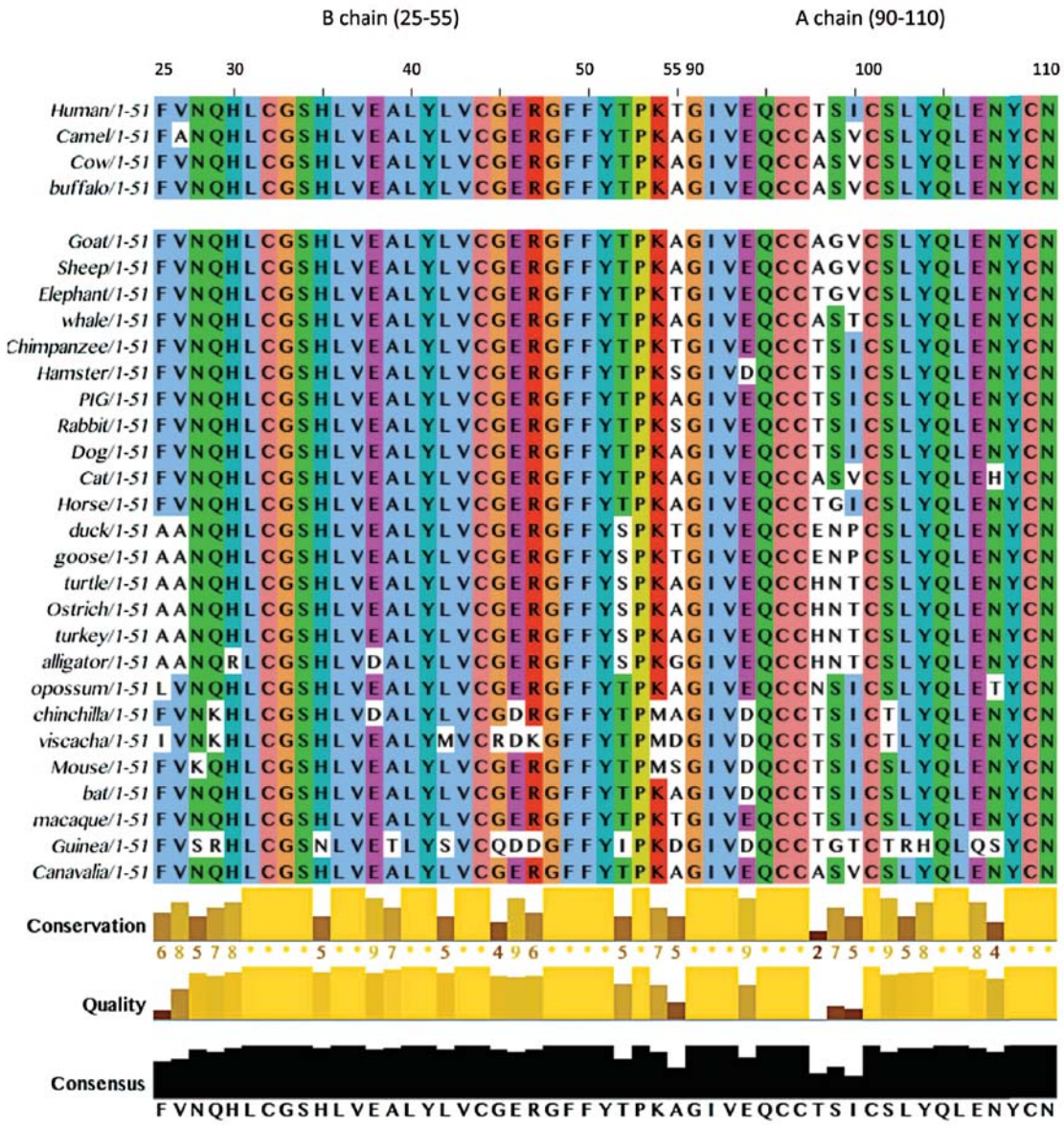

Figure 2. Sequence alignment of B and A chain of different types of insulin. Cow, buffalo and jack bean insulin are identical and very similar to camel insulin (V26A at B2). Residues 8-10 in the A chain and B30 and B1-2 residues in the B chain are least conserved in different types of insulin. Camel insulin differs from human insulin at B2, B30, A8 and A10 positions which are non-conserved sites.

original surface residues shown to be important in receptor binding, a cluster of residues (Ser101, Leu102, Glu106, His35, Glu38, and Leu42) known as the primary binding surface disrupt binding to receptor if mutated (27,28). It is worth noting that His10 (35 in Fig. 2) is involved in Zn coordination necessary for the hormone activity.

We analyzed the effect of mutations on the specific activity of insulin. Three relevant human insulin mutants are known (B: Val26Ala, Thr54Ala and A: Thr97Ala). Two mutations (B: Val26Ala and Thr54Ala) in the human insulin increase its specific activity by 110 and 102±21%, respectively, while the third mutation (A: Thr97Ala) decreases specific activity to 87±13% (29). Only one residue (Thr98 of A chain) out of three mentioned above interacts with insulin receptor (27). The mutations in the B chain terminals might have an impact on the conformational changes and stability of the hexamer and their conversion into active monomer.

All these amino acids are conserved in camel, water buffalo and bovine insulin. All three types of insulin are quite unique if compared with others that are identical in primates and vary by one or more amino acid in other species. There is no evidence of anti-diabetic properties of water buffalo and cow milk (30-32). Literature search on insulin from sheep and goat varying form, Thr54Ala, Thr97Ala, Ile99Val, by additional mutation Ser98Gly do not provide any evidence on anti-diabetic properties. We conclude that the camel insulin itself is most likely not responsible for anti-diabetic properties of camel milk.

Nano particles. Mucosal surfaces are frequent routes for delivering drugs to the body. Unfortunately, drugs such as peptides and proteins are unable to overcome the mucosal barriers and are degraded (by digestive enzymes if delivered orally) before reaching the blood stream. It provokes the question how insulin

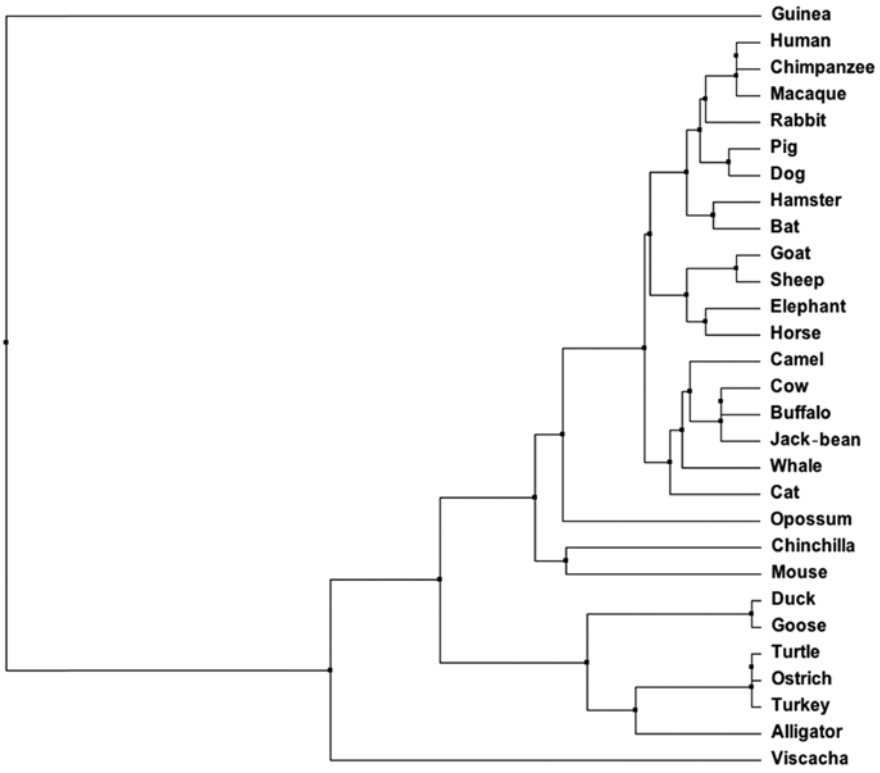

Figure 3. Phylogenetic analysis of camel insulin: Average distance tree shows evolutionary relationship among insulin of different types of organism. Camel insulin is grouped with cow, buffalo and jack-bean insulin. Human insulin is clustered with chimpanzee and macaque insulin.

		B chain				A chain		
		25 30	40	50	…	90	100	110
Human	P01308	FVNQH	LCGSHLVEAL	YLVCGERGFF	YTPKT	G	IVEQCCTSIC	SLYQLENYCN
Classical binding			V Y	GFF Y		G		Y N
Insulinopathy				SL				
Hexamer forming			L				L	
Primary binding		H E					SL E	

Figure 4. Functional amino acids of human insulin.

in the camel milk could be protected in the stomach to reach the target. Possible explanation may be hidden in the uniqueness of camel milk. Camel milk does not easily coagulate at low pH, it has good buffering capacity, has different proportions of caseines and fatty acids and makes larger lipid micelles than observed in milk of other mammals. It may be possible that insulin in the camel milk is encapsulated in the micelles and passes through the stomach to the intestine. For example when compared with the cow's milk: i) Kappa caseine micellar fraction reacting with clotting enzymes has different electro-potential and lower electrophoretic mobility and accounts for only ~5% of total casein vs. ~13.6% in cow, ii) The micellar size show the mean diameter of 280÷325 μm vs. 160 μm for cow (13), iii) Raw cow milk contains less insulin than camel and it loses even more in processing before reaching a diary store (12). There is evidence that size of lipid micelles becomes larger in milk of cows exposed to hot weather and water deprivation (33). In the desert climate camels are well adjusted to both such conditions, which might explain unique properties of their milk even during drought (34). If due to unique features of the camel milk, insulin is able to cross stomach and get absorbed efficiently into blood stream then 'camel milk-like features' could be used for the formulation of insulin for oral delivery in humans. We do not have evidence of insulin presence in micelles, although nanoparticles were used for oral delivery of proteins (35-37). Nafissi-Varcheh *et al* investigated biodegradable polyester polymers with different molecular weights and lactic/glycolic acids ratios in simulated gastrointestinal fluids. They intend to use microparticles for oral protein delivery. They reported that nanoparticles could be suitable for the preparation of protein-loaded microspheres (35).

Prego *et al* used the mucoadhesive polysaccharide chitosan nanoparticles, chitosan-coated oil nanoparticles and chitosan-coated lipid nanoparticles showing significant capacity for the association of proteins such as insulin, salmon calcitonin and other proteins. They showed that chitosan-coated nanoparticles exhibited capacity to enhance the intestinal absorption of the model peptide, salmon calcitonin, and long-lasting decrease in the calcemia levels in animals (36). Vila *et al* developed new biodegradable polymer nanoparticles: poly(ethylene glycol) (PEG)-coated poly(lactic acid) (PLA) nanoparticles, chitosan (CS)-coated poly(lactic acid-glycolic acid (PLGA) nanoparticles and chitosan (CS) nanoparticles. These were

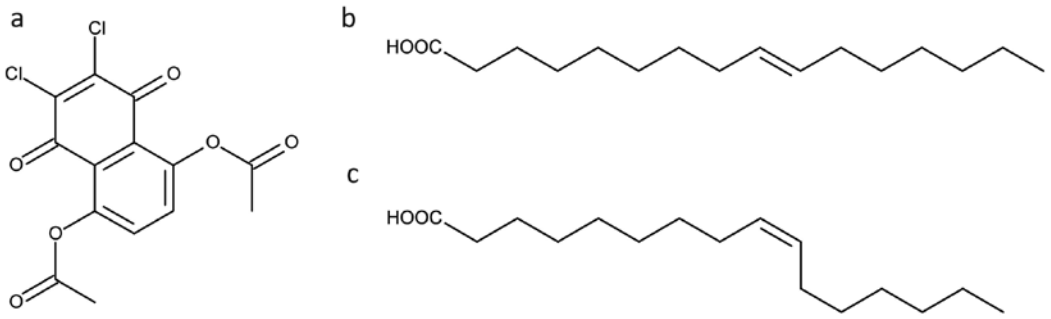

Figure 5. Insulin-like small molecules: (a) 5,8-diacetyloxy-2,3-dichloro-1,4-naphthoquinone; (b) trans-palmitoleate (trans-16:1n-7); (c) cis-palmitoleic acid.

tested successfully to load proteins, and to deliver them in an active form to transport them across intestinal mucosae (37).

Insulin-like small molecules. He *et al* developed an *in vitro* screening assay searching for insulin mimetics. Screening the small molecule chemical libraries, they found a compound (5,8-diacetyloxy-2,3-dichloro-1,4-naphthoquinone, Fig. 5a) that activates insulin receptor directly binding to the receptor kinase domain, to trigger its kinase activity sensitizing insulin's action. Drug was delivered orally to wild-type C57BL/6J mice and db/db (diabetic) and ob/ob (obese) mice and it was shown to elevate glucose uptake in adipocytes (38).

Mozaffarian *et al* investigated over 3700 adults in the Cardiovascular Health Study to determine if trans-palmitoleate (trans-16:1n-7, Fig. 5b) was related to new-onset diabetes. An endogenous cis-palmitoleic acid (Fig. 5c) (of adipose or hepatic source), could be beneficial protecting against insulin resistance but also harmful causing cardiovascular risk in humans. Contrary, trans-palmitoleic was associated with lower incidence of diabetes. The individuals taking it had a much lower risk of developing diabetes; ~60% lower risk among participants in the highest quintile (39). Trans-palmitoleate is strictly exogenous and naturally, occurring in dairy/ruminant trans-fats. It is worth noting that among long chain fatty acids present in camel milk C16 and C18 dominate with C16 on par to cow milk in saturated category but ~3 times higher for unsaturated C16:1 (39). This might further support the anti-diabetic benefits of drinking camel milk. Additionally, substantial work has been carried out in plants, as reviewed below.

Insulin and insulin-like molecules in plants. Traditional holistic practitioners in many different parts of the world recommend the consumption of plants variety for regulation of glycaemia (40-46). There are also more systematic approaches to evaluate anti-diabetic activity of food. Broadhurst *et al* examined the possible effects of 49 herbs, spices, and medicinal plant extracts on the insulin-dependent utilization of glucose using a rat epididymal adipocyte assay. They found that cinnamon was the most bioactive product followed by witch hazel, green and black teas, allspice, bay leaves, nutmeg, cloves, mushrooms, and brewer's yeast (43). However, no particular active chemicals were identified.

Varieties of legumes were reported to have anti-diabetic properties (47-50). Bean pods (Phaseolus vulgaris) are among the most used traditional remedies with anti-diabetic activity. To be effective, fairly high doses of aqueous extracts need to be given. There is no clear evidence what the active ingredient is. However, authors suggest that by α-amylase inhibitory effect, beans might be effective in preventing or ameliorating type 2 diabetes (48). Nevertheless, beans similarly like camel milk contain insulin or insulin-like protein sequences. Soon after discovery of pancreatic insulin in early 1920s, insulin-like protenaceous material was found in many plants (bean, lettuce, onion and beat). In the 1970s and 80s, several research groups have isolated and well characterized insulin-like protenaceous material and found that it exhibits same hypoglycemic activity, identical molecular weight, chromatographic and immunological properties. In 2003, high level (50 mg insulin/100 g protein = ~1000 units insulin/100 g protein) of insulin-like substance from legume *Vigna unguicultata* (cowpea) was detected.

Of note, sequence of cowpea insulin-like material was identical to bovine insulin and similar to human insulin (three mutations at Thr54Ala in the B chain and Thr97Ala and Ile99Val in the A chain as shown in Fig. 2) and camel insulin (just one mutation at Val26Ala in the B chain). Presence of insulin in plants is disputed by biologists despite the fact that insulin was proven to be present in beans and beans supplemented with insulin/glucose were able to accelerate *Canavalia ensiformis* (Jack bean) seedling development (51,52). Additionally, Xavier-FilhoI *et al* reported that proteins associated with insulin signaling pathways in vertebrates are also present with insulin-like molecules in plants (52). This raises question if consumption of insulin containing beans can alleviate symptoms of diabetes. It seems to be very unlikely due to the fact that most beans are consumed boiled that would denature proteins.

The other possibility is presence of other molecules that can have drug-like properties. For example α-amylase is an enzyme that hydrolyses α-bonds of large polysaccharides, such as starch and glycogen, yielding glucose and maltose (53,54). Inhibitors of α-amylase are oral anti-diabetic drugs that reduce the impact of carbohydrates on blood sugar.

Reducing excessive intake of refined carbohydrates plays an important role in prevention of obesity and type 2 diabetes mellitus. Tormo *et al*, studied purified pancreatic α-amylase inhibitor from white beans (*Phaseolus vulgaris*) that was administered orally for 22 days to non-diabetic and type 2 diabetic Wistar rats. α-Amylase inhibitor from that bean significantly reduced glycaemia in the ND and diabetic animals (55). Two other reports strongly support these findings about anti-diabetic effects of α-amylase inhibitors from beans (47,56).

Controlled clinical experiment of camel insulin on diabetic patients showed that regular consumption of camel milk lowered blood glucose level and in 25% of patients additional insulin requirement was reduced. It is contrary to the results of insulin therapy on the diabetic patients. Once insulin therapy starts, patient has to take insulin lifelong and generally insulin dose keeps on increasing with time. It seems that camel milk delivers insulin in a different form (than in other mammals) and/or provides some other compound in addition to insulin that improve the health of diabetic patients.

Sequence of camel insulin and its predicted digestion pattern do not suggest differentiability to overcome the mucosal barriers before been degraded and reaching the blood stream. However we cannot exclude the possibility that insulin in camel milk is present in nanoparticles capable of transporting this hormone into the blood stream. Although, much more probable is that camel milk contains 'insulin-like' small molecular substances that mimic insulin interaction with its receptor.

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