International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 1 | JAN-MAR| 2018 | 74-81
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

THE STIMULATORY EFFECT OF STRYCHNINE ALKALOID ON PRODUCTION OF GLUCAGON LIKE PEPTIDE-1 HORMONE (GLP-1) IN THE GUT OF ALLOXAN INDUCED DIABETIC RABBITS

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Experimental Work Done At:
Chemical Analysis was done in R.V Labs, Guntur, Andhra Pradesh state, India.
Research work on rabbits was done in Ranchi Veterinary College, Rabbit Farm Unit, Kanke, Jharkhand, India.
The experimental protocol has been approved by the institutional animal ethics committee, proposal number being IAEC.049/2011.

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ABSTRACT

The Present investigation was carried out to study the stimulatory effect of aqueous extract of strychnine alkaloid on production of gastrointestinal incretin hormone called Glucagon Like Peptide-1(GLP-1) hormone in the gut of alloxan induced diabetic rabbits. The stem piece weighs about 15 grams of Strychnos-nux-vomica plant was soaked in 150 ml of normal water over night for 12 hours, 0.720 mg alkaloid containing aqueous extract was obtained. When the aqueous extract of this safety dosage Srychnine alkaloid solution was orally given along with the carbohydrate containing food to the rabbits, the hypoglycemic effect was observed with increased levels of plasma insulin secretions in the alloxan induced diabetic rabbits. It was due to the stimulatory effect of strychnine alkaloid on the intestinal L –cells which further produced high concentrations of Glucagon like Peptide Hormone-1 (GLP-1) from the L-cells. Later the GLP-1 hormone absorbed from the intestine and enters the circulatory system and activated the beta cells of pancreas to produce insulin. The insulin hormone naturally decreases the blood sugar level and leads to hypoglycemia. Since GLP-1 is destroyed by Dipeptidyl peptidase-4 (DPP-4) enzyme present in blood, GLP-1 has very less half-life period, 1.30 to 1.45 minutes. To overcome this problem, an additional oral drug Sitagliptin, an inhibitor of Dipeptidyl peptidase-4 was given regularly to alloxan induced diabetes Rabbits. Simultaneously, the alkaloid delays the rate of absorption of digested food at the gut which all the treatments leads to hypoglycemia in the diabetic rabbits.

KEY WORDS

Incretin hormone, Glucagon like Peptide Hormone-1 (GLP-1), Strychnine alkaloid, Dipeptidyl peptidase-4, Type 2 diabetes, Neogenesis

INTRODUCTION:

Strychnos-nux-vomica Linn (Loganiaceae) is a medium sized tree, both wild and cultivated, throughout the India. The plant is popularly known as Snake wood in English. In the Indian system of medicine, the medicinal attribution of this species has been known for long time.

As per the traditional claims, the root bark is used in cholera, leaves used in chronic wounds and ulcers, and seeds used as appetizer, antiperiodic, purgative, asthma, diabetes, skin diseases etc. In pharmacology, only few activities such as analgesic, apoptotic effect, antidepressant antidote for snake poisoning antitumor has been proved. Hence the present study was undertaken to evaluate the potential production of GLP-1 by stimulation of strychnine alkaloid in the gut for treating diabetic rabbits.

Glucagon Like Peptide-1 is a hormone that is encoded in the proglucagon gene. It is mainly produced in enteroendocrine- L cells of the gut and is secreted into the blood stream when food containing fat, protein hydrolysate and/or glucose enters the duodenum. Its particular effects on insulin and glucagon secretion have generated a flurry of research activity over the past twenty years.

Molecular level investigations on GLP-1:

Drucker and co-workers initially demonstrated the effect of GLP-1 on increasing insulin mRNA levels in 1987 [1]. In 1992 Fehmann and Habener showed that GLP-1 (10 nM) treatment induced the proinsulin gene using a chloramphenicol-acetyltransferase (CAT) reporter gene assay, and it increased insulin mRNA levels and insulin content in the βTC-1 cell line following 24 hr of treatment [2].

In 1995 it was shown that prolonged treatment of rat insulinoma cells with GLP-1 (1 or 10 nM for 24 hr) resulted in a 1.5-fold increase in intracellular insulin [3]. Use of the general transcription inhibitor actinomycin D and the protein synthesis inhibitor cyclohexamide showed that the increase in insulin transcription and consequently insulin translation accounted for the increase in insulin content. However, the effect of actinomycin D inhibition did not completely eliminate the GLP-1-induced increases in the levels of insulin transcript. This was the first evidence of an important role for stabilization of the insulin transcript in the GLP-1- mediated increase in intracellular β cell insulin levels, at least in insulinoma cells, during prolonged treatment. By contrast actimomycin D treatment did significantly reduce the effect of GLP-1 upon induction of GLUT1 and hexokinase I genes. Thus, it became apparent that the beneficial effects of GLP-1 on insulin secretion arose from the stimulation of transcription in the β cell as well as enhancement of acute insulin secretory responses to glucose.

The ability of GLP-1 to induce transcription of the insulin gene was later demonstrated using a luciferase reporter gene assay for the rat insulin I gene in INS-1 cells [4] where a maximum 2-fold increase in luciferase activity was noted. More recently similar results were also obtained when the luciferase-linked human insulin promoter was transfected into INS-1 cells [5].

Clinical investigations on GLP-1:

Nutrient ingestion is the primary physiological stimulus to the L-cell and results in a biphasic pattern of GLP-1 secretion. An initial rapid rise in circulating GLP-1 levels occurs 15–30 min after a meal, followed by a second minor peak at 90–120 min [6]. The initial rapid rise in GLP-1 secretion must be mediated indirectly, through a neuro/endocrine pathway, rather than through direct interactions of the luminal contents with L-cells [7]. Glucose and fat have been found to be potent stimulators of GLP-1 secretion when ingested [8].

The Glucagon Like Peptide- 1 plays so many crucial roles in normal and diabetic subjects. In 1992 Exogenous administration of GLP-1 as a continuous intravenous infusion at a dose rate of 0.75 pmol/kg/min was anti-diabetogenic in both type 1 and type 2 diabetic subjects [9]. It lowered fasting and post-prandial glucose levels in type 2 diabetic patients because it increases insulin secretion and decreases glucagon secretion, as well as delaying gastric emptying.

Further studies showed that the effects in humans were consistent [10] [11][12][13]. Furthermore, GLP-1 was capable of lowering blood glucose even in patients with long-standing and severe T2DM and even in patients who no longer responded to sulfonylureas. In 1986 and 1987 GLP-1 was shown to have insulin tropic properties in rodents [14].

GLP-1R−/− mice display abnormally high blood glucose levels after an intra peritoneal glucose challenge demonstrating that GLP-1 is important for clearance of the glucose load, irrespective of the site of glucose entry into the circulation [15]. GLP-1R activation either *in vitro* in ductal or acinar cell lines or *in vivo* in rodents causes an initial burst of proliferation followed by cell cycle arrest leading to differentiation of a large fraction of these cells into pancreatic hormone expressing cells [16].

Bulotta and colleagues in the Perfetti laboratory quantified increases in cell number and cell cycle distribution in a rat pancreatic ductal cell line, ARIP, treated with GLP-1 (10 nM for 12 hr, 24 hr or 48 hr)

following induction of cell cycle arrest [17]. Fehmann and colleagues were the first to study the effect of GLP-1 on pancreatic acinar secretions [18]. Matsumura and colleagues [19] have shown that GLP-1 decreased cAMP and suppressed glucagon secretion in INR1-G9 cells. In low levels of glucose and FFA, fat storage is an important source of energy for β cells [20].

GLP-1 has been shown to stimulate fatty acid synthesis from triglyceride stores in both clonal β cell lines and in rat islets. New observations related to gut factors and the control of β cell mass have recently been made in patients following bariatric surgery, implicating endogenous GLP-1 as a possible pathogenic factor [21]. Current therapy of type 2 diabetes includes lifestyle modifications, such as diet and exercise, and the use of a variety of pharmacological agents that target increase insulin secretion, decrease hepatic glucose production, and increase insulin action. Despite these approaches, a number of type 2 diabetic patients may require exogenous insulin. Facilitation of type 2 diabetes treatment may be obtained through beta- cell transplantation or, on a more prospective basis, beta-cell mass expansion after stimulation of beta -cell regeneration/neogenesis in diabetic patients. Indeed, the emerging understanding of beta -cell growth in the adult from precursor cells found in the pancreatic ducts holds the promise of developing new strategies for stimulating beta- cell regeneration. Such an approach may involve the delivery of appropriate growth factors to these progenitor cells to obtain a full beta- cell phenotype. Glucagon Like Peptide-1 (GLP-1) could be one of the most promising candidate for doing so.

The gastrointestinal incretin hormone Glucagon like Peptide Hormone-1 (GLP-1has been repeatedly reported to affect beta-cell function, replication, apoptosis, and neogenesis (reviewed in Refs. 25, 28). GLP-1 engages a specific G-protein coupled receptor that is present in tissues other than the pancreas (brain, kidney, lung, heart, major blood vessels). The most widely studied cell activated by GLP-1 is the insulin-secreting beta cell where its defining action is augmentation of glucose-induced insulin secretion. Upon GLP-1 receptor activation, adenyl cyclase is activated and cAMP generated, leading, in turn, to cAMP-dependent activation of second messenger pathways, such as the PKA and Epac pathways. As well as short-term effects of enhancing glucose-induced insulin secretion, continuous GLP-1 receptor activation also increases insulin synthesis, and beta cell proliferation and neogenesis. Although these latter effects cannot be currently monitored in humans, there are substantial improvements in glucose tolerance and increases in both first phase and plateau phase insulin secretory responses in type 2 diabetic patients treated with exendin-4.

Since its discovery, GLP-1 has received much attention as a possible new treatment for type 2 diabetes. GLP-1 stimulates insulin secretion and biosynthesis and inhibits glucagon release, both of which is glucose dependent and therefore represents a safe way of lowering increased blood glucose. One of the major drawbacks to the use of the native peptide in the clinic is its rapid degradation in serum due to the presence of a Di Peptidyl Peptidase-IV (DPP-IV, also known as CD26) recognition site in the N-terminus [22]. It is the key factor limiting the therapeutic potential of GLP-1. This enzyme, present in the blood stream and on cell membranes, cleaves GLP-1 (7-36) peptide to yield the inactive GLP-1 (9-36) form. GLP-1 hormone half-life period is just 1.30 -1.45 minutes. Therefore, many modifications have been made to GLP-1 to increase its biological half-life and consequently its efficacy *in vivo*. Exendin-4 (Ex-4, also called exenatide), sitagliptin (MSD & Co, Mumbai), berberine a GLP-1R agonist is now available for treating type 2 diabetes mellitus (T2DM).

From this one can understand GLP-1 is playing a great role in diabetic patients in decreasing the blood sugar level by stimulating the pancreas, so that the effect of strychnine alkaloid on GLP-1production is considered in this work.

MATERIALS AND METHODS:

Plant material

Strychnous nux vomica stem pieces were collected from Srisailam, one of the great shrines of Lord Shiva, located in Nallamala forest, Kurnool district, Andhra Pradesh state, South India. Botanical identification was done by Prof. D. Durgaiah, Director, Plant Anatomy Research Centre, Medicinal plant research unit, Hyderabad, Andhra Pradesh.

Procedure for the aqueous extraction of alkaloid strychnine:

Though the strychnine alkaloid is insoluble in water, it has partial soluble nature in water. Based on this principle, the stem pieces weigh about 15 grams were soaked in different vessels containing 150ml normal drinking water over night/12 hours at room

temperature. The stem pieces were then taken out and the aqueous extracts were ready for quantitative analysis of the alkaloid, Strychnine.

The extracts were prepared freshly whenever required and the stem pieces were reused repetitively in each context.

Quantitative analysis:

Colorimetric method:

The concentrations of the strychnine alkaloid are measured by using the Colorimetric method.

Alloxan induced diabetes rabbits:

New Zealand white male and female rabbits weighing about 1 to 1.5 Kg were used in this study. The rabbits were maintained under standard laboratory conditions at 25±2ºC, relative humidity 50±15% and normal photo period (12 hr dark and 12hr light) were used for the experiment. The young animals were placed in a cage with no food for 24 hours before experiment. Only the most definitely healthy animals were taken.

Animals were grouped according to the approximately same plasma glucose level and body weight. The New Zealand rabbits were divided into four groups:

Group I: Normal control rabbits (no -4) received normal saline water (no-4)

Group II: Diabetic control rabbits (no-4) orally received Alloxan (120mg/kg) and Sitagliptin (100 mg)/day

Group III: Diabetic rabbits (no -4) orally received standard drug Glipizide (0.5mg/kg)/day and Sitagliptin (100 mg)/day for four weeks from the fourth day of Alloxan treatment.

Group IV: Diabetic rabbits (no -4) orally received aqueous extract of *Strychnos nux vomica stem* (100 ml/kg)/day and Sitagliptin (100 mg)/day for four weeks from the fourth day of Alloxan treatment.

Diabetes was induced in all the groups of rabbits except normal control group. Diabetes mellitus in rabbits were induced by intra peritoneal injection of Alloxan 120 mg/kg dissolved in 1% citrate buffer (pH 4.5) in either sex of New Zealand rabbits, fasted for 24 hours. Animals were considered to be diabetic if they had plasma glucose level of greater than 260 mg/dl in addition to polyuria, hyperphagia and decrease in body weight.

From the next day onwards, rabbits were fed normally for 4 days. Then 5th day onwards, the day 1 count started for the experiment. The three groups (**II, III and IV**) of alloxan induced diabetes rabbits received daily Sitagliptin (100mg), an inhibitor of Dipeptidyl peptidase-4 (DPP-4) to enhance the activity of GLP-1 hormone activity. Group-III rabbits received standard drug Glipizide (0.5mg/kg) dialy along with normal diet and normal drinking water. Group- IV rabbits received test sample solution, 150 ml of aqueous extract of strychnine solution/day instead of drinking water and Sitagliptin (100mg) daily but diet was same. The animals were administered daily on this supplement for 30 days. On day-1(0 week), day-8(1st week), day-15(2nd week), day-22(3rd week) and day-29(4th week) 5 ml of blood sample was withdrawn from animals through lateral saphenous vein under anaesthetized condition and collected in tubes containing potassium oxalate and sodium fluoride as anticoagulant. 2.5 ml of blood used for serum glucose estimation and 2.5 ml was used for serum insulin estimation. Serum glucose level was determined by commercially available GOD- POD Kit using auto analyzer and serum insulin was estimated by ELISA test.

At the end of experiment rabbits were fasted overnight and scarified by cervical decapitation. Blood is collected; plasma and serum were obtained and used for determination of various biochemical parameters like Blood glucose level, total protein, Total cholesterol, Serum creatinine & Blood urea nitrogen. The liver was carefully removed, homogenized and the homogenate was used for the estimation of glycogen level.

It was found that the strychnine aqueous extract received group of rabbits and standard drug Glipizide received group of rabbits show all most all similar reduction of both blood sugar levels and insulin levels. No side effects were found in both strychnine alkaloid consumed and Glipizide rabbits, from this we can understand the aqueous extract of strychnine alkaloid is showing the same hypoglycemic effect and high insulin production as well as Glipizide by inducing the more production of GLP-1 hormone by stimulating the L-cells of the gut.

Statistical analysis:

The values of blood sugar and serum insulin tests were expressed as mean ± SEM. Student's t-test was used to analyze statistical differences between the mean of the various groups. Microsoft Excel 2007 was used for statistical calculations. Mean values were considered significantly different if $P < 0.05$, $P < 0.01$ and $P < 0.001$.

RESULT:

From the Table-1 and Table-2, the above experiments are revealing that the alloxan induced diabetic rabbits

showing hypoglycemic effect through the increased secretions of Insulin hormone with oral administration of a safe dosage of aqueous extract of strychnine alkaloid solution.

In the Group- I normal control rabbits, since the pancreas was working properly, and insulin secretions were within the normal range, the blood sugar level had not shown much variations until the end of the day (29 days) of the experiment.

In the Group-II, alloxan induced diabetic control rabbits, the pancreas was damaged by the drug so that the insulin hormone secretions were too low by the end of the experiment and blood sugar levels were also too high than the normal range.

In Group-III, alloxan induced diabetic rabbits, standard drug Glipizide was given. The blood sugar levels were regulated below the normal range and more insulin levels were observed. It is showing the hypoglycemic effect of Glipizide, a standard drug for diabetes and due to its stimulatory effect on L- cells of gut, more GLP-1 hormone was secreted. It increased insulin level.

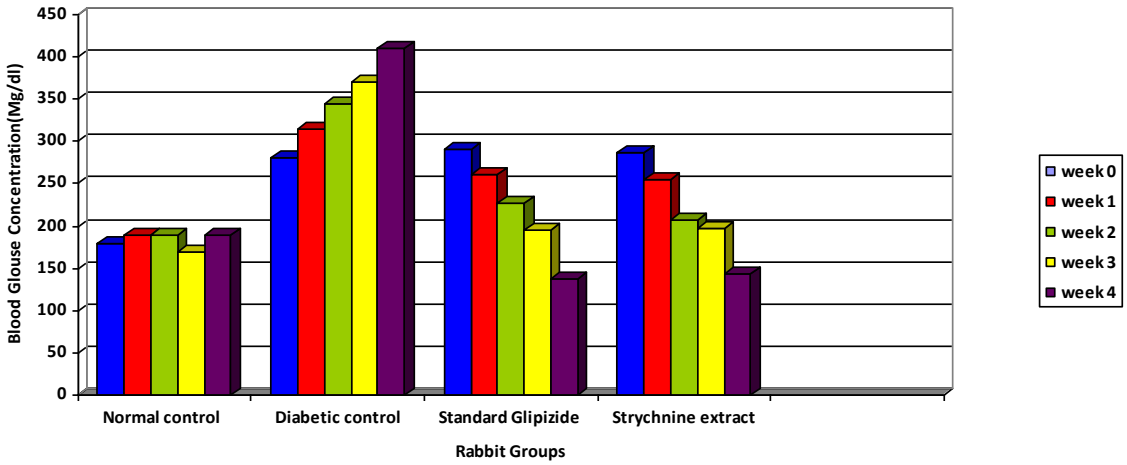
In Group-IV, alloxan induced diabetic rabbits, the test solution aqueous extract of Strychnine alkaloid was given orally, and hypoglycemic effect was observed along with increased insulin production. Initially the rabbits had only 22 µ U /ml of serum insulin but by the end of the experiment the level was increased to 52 µ U /ml and blood sugar was also reduced in a remarkable concentration.

From this result one can understand that the safety dosage of aqueous extract of strychnine alkaloid is stimulating the L –cells of the gut and secreting more GLP-1 hormone and it is absorbed and stimulating the pancreatic cells to produce more insulin hormone (graph-2) and blood glucose levels are remarkably decreased(graph-1). Sitagliptin administration showed its inhibitory effect on Di Peptidyl Peptidase-IV (DPP-IV) which destroys GLP-1, so that GLP-1 extended its action on pancreas. The challenge of very short half-life period of GLP-1 in blood has overcome

Table-1 Effect of aqueous extract of Strychnous-nux- vomica stem on the blood glucose level (BGL-mg/dl) in Alloxan induced diabetic rabbits

S. No	Groups	0 week (day-1)	1 week (day-8)	2 weeks (day-15)	3 weeks (day-22)	4 weeks (day-29)	Difference between 4th and 0 week in blood sugar
1	Normal control	180 ± 5	190 ±15	190 ±10	170±5	190 ±5	↑10
2	Diabetic control	280 ± 4	315 ±10	345 ±15	370 ± 15	410 ± 10	↑130
3	Standard	290 ± 8	260 ± 5	227 ± 8	195 ± 4	138 ± 5	↓152
4	Aqueous extract	286 ± 5	255 ± 7	207± 3	197± 8	143 ± 6	↓143

Normal blood sugar level of rabbit is from 190mg/dl to 260 mg/dl. Each value is represented as mean± SEM, No. of animals (n) = 16, **p< 0.01 Vs Normal control, ££ p<0.01 Vs Diabetic control, one-way ANOVA followed by Dunett's Test

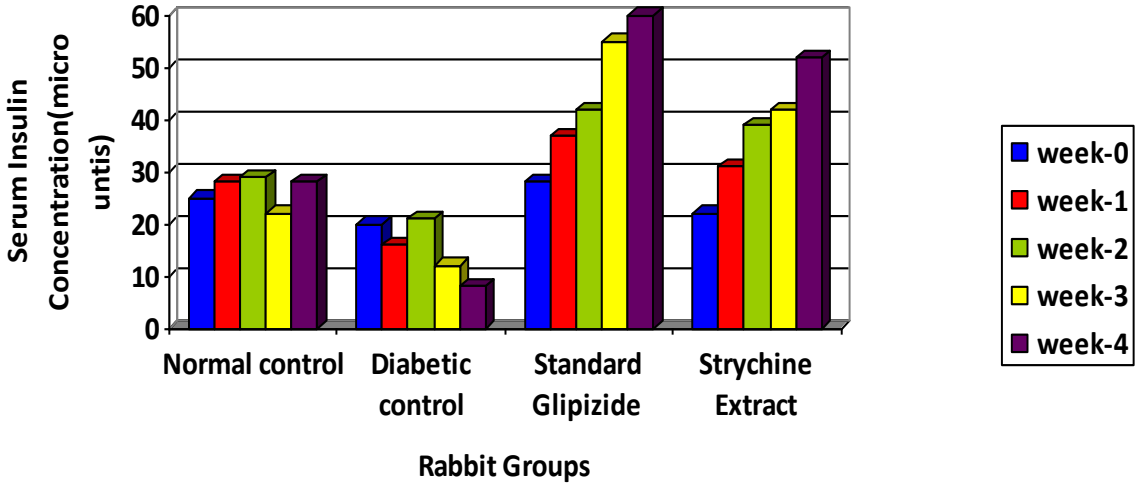


Graph-1 - Effect of aqueous extract of strychnine alkaloid on the blood glucose level (BGL-mg/dl) in Alloxan induced diabetic rabbits

Table-2 Effect of aqueous extract of Strychnous-nux- vomica stem on the serum insulin level (μ U /ml) in Alloxan induced diabetic rabbits

S. No	Groups	0 week (day-1)	1 week (day-8)	2 week (day-15)	3 week (day-22)	4 week (day-29)	Difference between 4th and 0 week in serum insulin
1	Normal control	25	28	29	22	28	↑ 03
2	Diabetic control	20	16	21	12	08	↓ 12
3	Standard Glipizide	28	37	42	55	60	↑ 32
4	Aqueous extract (Strychnine)	22	31	39	42	52	↑ 30

Normal serum insulin level of rabbit is from 10 -50 μU/ml. Each value is represented as mean± SEM, No. of animals (n) = 16, **p< 0.01 Vs Normal control, ££ p<0.01 Vs Diabetic control, one-way ANOVA followed by ELISA Test



Graph-2- Effect of aqueous extract of Strychnine alkaloid on the serum insulin level (μ U /ml) in Alloxan induced diabetic rabbits

DISCUSSION:

The insulin hormone is responsible for the transport of the glucose through the cell membrane so that the cells get fuel for the production of energy through cellular respiration. The sole source of insulin is pancreas, a mixed gland in mammals. The beta cells of islets of Langerhans of pancreas secrete insulin hormone according to the blood sugar levels automatically. When blood sugar levels are increasing, the pancreas produce more insulin to regulate the sugar level by allowing the glucose in to the cells or by glycogenesis on liver. In the case of diabetic patients, the pancreas lost its capacity to secrete sufficient amounts of insulin hormone due to damage of pancreatic cells so that blood glucose levels increase and leads to diabetes.

Now a day's many drugs and phyto medicines are available to enhance the pancreas activity. Biguanides (Metformin), Sulfonylureas (Tolbutamide, Glipizide, Glimpiride) Thiozolidinediones (Pioglitazone) etc.. Along with that at many places, diabetes patients have been preferring herbals for reducing the blood sugar level.

The medicines or the herbal treatments function in various ways to control blood sugar level. Some stimulate pancreas, some other decrease appetite, some more stimulate L-cells present in the gut so that an incretin hormone called GLP-1is secreted and absorbed and stimulate pancreatic beta cells. The beta cells then produce more amount of insulin hormone. Insulin hormone permits glucose to pass through the

plasma membrane of the cells. The blood sugar level reduces.

There is another enzyme present in blood Di Peptidyl Peptidase-IV (DPP-IV) hydrolyses GLP-1, so that GLP-1 could not prolong its action. In general, within 1.30 to 1.45 minutes the GLP-1 gets destroyed, sothat there would be no further action of GLP-1 on pancreas. In view of continue the GLP-1 hormone action, an inhibitor sitagliptin is used to inhibit the action of Di Peptidyl Peptidase-IV (DPP-IV) over GLP-1.

Now the GLP-1destruction is delayed, insulin production is continued, blood sugar level is reduced. It is similar to metformin mechanism. Metformin also stimulate L-cells of gut to produce GLP-1 hormone. It additionally play the role in regenerating the pancreatic cells which is not possible by alkaloid.

The benefit of using aqueous extract is that, it delays emptying stomach so that the quantity of intake food will be reduced which is another advantage of less input of food to the body. All this occur only when the safety dosage of strychnine alkaloid extract is consumed. This stem piece is very cheap and available across the forests. The way of extraction of alkaloid is also so easy and simply soaking 15grams of stem piece in 150 ml drinking water over night. It contains nearly 0.720 mg strychnine alkaloid which is a safe dosage for the 1.3 - 1.5 kg rabbit. The stem piece we can use it repeatedly for 30- 40 nights. Later a new stem piece must be used because the concentration of the alkaloid decreases upon continuous usage.

CONCLUSION:

When safety dosage of aqueous extract of strychinine alkaloid solution is given orally to the alloxan induced and sitagliptin injected diabetes rabbits have shown very good improvement in plasma insulin levels and reduced blood sugar levels**.**

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