Toxicological Pathologic Study of effect of various Insulin doses in mice

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Summary

This study concentrate on the effect of synthetic insulin in laboratory animals, 80 white mice, randomly divided into four main groups of males and females control – untreated, treated as low dose, intermediated dose and high dose, then every group also divided in two subgroups, the first subgroup was given Actrapid and second subgroup was given Monotrade , the tested material was given daily subcutaneously untreated control group .

The injection was continued until animals were killed after 24 weeks of treatment .Clinical, macroscopical and microscopical studies of experimental mice in comparison with the control group and the result were as follows :

1- Injected mice showed normal activity, with increase body weight and low food consumption in comparison to controls. On several occasions, there were attacks of hypoglycaemia with coma in treated mice.
2- Macroscopic examination of treated mice showed presence of several layers of adipose tissue around visceral organs , in peri renal region , in the serosal areas of small intestine , in peri bronchial region, in subcutaneously , peri pancreatic region , splenic enlargement in scattered treated animals ,
3- Histopathological changes were seen as fatty infiltration / degeneration in the liver, dilated cortical tubules in the kidney and enlarged zona fasciculate of the adrenals. In addition to atrophy and / or depletion of pancreatic islet of langerhans, also extramedullary haemopoiesis and / or atrophy of lymphoid tissue in the spleen, with peri follicular deposits of amyloid in Splenic white pulp and in glomeruli of kidneys of group B. Furthermore, there were evidence of steatitis (focal inflammation of adipose tissue) and enlargement of zona fasiculata in adrenals.

دراسة مرضية سمية لتأثير الانسولين بجرع مختلفة في الفئران

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الخلاصة

أجريت الدراسة لمعرفة تأثير الانسولين الصناعي في الحيوانات المختبرية وقد شملت 80 فأر أبيض ، قسمت عشوائياً الى اربع مجموعات رئيسة (ذكور وأناث) وبدورها وزعت الى مجموعتين ثانويتين حقنت المجموعة الثانوية الاولى الى عقار Actrapid والمجموعة الثانوية عقار Monotrade تحت الجلد وبمعدل يومي مع ترك مجموعة واحدة ، حيوانات سيطرة . استمر الحقن حتى قتل الحيوانات وذلك بعد مرور اربعة وعشرون اسبوعاً . أجريت الفحوصات السريرية والعيانية والمجهرية للحيوانات المحقونة فضلاً عن مجموعة السيطرة وأظهرت النتائج الاتية :

1- أظهرت الفئران المحقونة حيوية ونشاط طبيعي مع زيادة معدل اوزانها وقلة استهلاك العلف مقارنة بمجموعة السيطرة فضلاً عن تعرض بعض الفئران الى نوبات هبوط السكر في الدم تحت الطبيعي .

2- أوضح الفحص العياني للفئران المعاملة وجود طبقاتعديدة من النسيج الشحمي حول اعضاء الجسم وتحت الكلى ، وفي الطبقة المصلية للامعاء الدقيقة وفي الرئتين والبنكرياس وتحت الجلد ، مع تضخم الطحال في الفئران المتعرضة الى نوبات متكررة لهبوط السكر في الدم .

3- تميزت التغييرات المرضية بوجود تنكسات دهنية ، وارتشاح الكلايكوجين داخل الخلايا الكبدية مع توسع في بعض النبيبات الكلوية وتضخم في الطبقة الحزيمية للغدة الكظرية ، فضلاً عن ظمور خلايا الجزيرات داخل البنكرياس وارتشاح الطحال بخلايا العدلات غير الناضجة والخلايا العملاقة مع نفاذ لمفاوي شديد للب الابيض وكذلك ترسب مادة النشوان في الطحال والكلى لفئران المجموعة B فضلاً عن وجود طبقات عديدة من النسيج الدهني (وارتشاح قسماً منه بخلايا التهابية) تحت الكلى ، الطبقة المصلية للامعاء الدقيقة والرئتين والبنكرياس وتحت الجلد والأعضاء التناسلية .

Introduction

Diabetes mellitus is a wide spread disease , all over the world in many countries , it was estimated that up to 300 million people suffer from this disease , mostly due to over feed and lack of proper exercise , in addition , that it is one of the hereditary disease , the number expected to be increase in 2025, especially

in developing countries , as it is expected that it will be up to 75 % because of the speed of social changes , as with the advance modern technology (1,2).

Diabetes mellitus divided in two types, insulin dependent Diabetes mellitus (IDDM) and non-insulin dependent Diabetes mellitus (NIDDM). IDDM (Juvenile-onset) mostly affecting Juvenile and young adult while NIDDM (adult – onset) affecting adult (3, 4). Insulin hormone was considered as the key enzyme in the intermediary metabolism and it is white small protein molecule, it has important effect on metabolism of carbohydrates, fats, minerals and proteins (5).

There are 2 kinds of synthetic insulin; the first as short acting and the second long acting, the first is watery clear, colourless solution the second one turbid, non pure solution, due to presence of zinc ions, both kinds of animal origin (2). Diabetes mellitus characterized by hyperglycaemia due to lack of insulin secretion or unresponsive effect of insulin especially in cases of decrease activation of beta cells to secrete insulin, was due to destruction of beta cells due to auto immunity (6, 7).

Hyperglycaemia in diabetes could cause serious complications as chemical changes progressing to retinopathy , nephropathy and neuropathy (8,9,10,11) and others noted that hyperglycaemia in pregnant mother caused pathological changes in heart and skeleton of the newborn (12).The present research was decided to :

1- Study pathological changes accompany using 2 kinds of synthetic insulin in different doses for limited times.
2- Study pathological changes associated with unnatural repeated hyperglycaemic attacks.

Material and Methods

1-Laboratory animals and housing:

In this experiment, there was 80 white mice, their age around 6-8 weeks and their body weight about 23 g .Mice were housed in clear plastic cages with five mice per sex in each cage, after washing and disinfecting the cages with 10 % sebtol, these cages were put in special room of the laboratory animal house which belong to the Veterinary Medical college .Mice were feed pellets from compressed wheat bought from Plasma and Vaccine institute of Ministry of Health.

2-Insulin dose:

In this study used 2 kinds of synthetic insulin , the turbid insulin Monotrade HM which was known as zinc suspension , Long acting formed of human insulin with sodium acetate , Sodium chloride and zink acetate , hydrochloric acid , Sodium hydroxide and water .

While the second kind was dissolved clear insulin Actrapid HM, which was short acting, insulin human watery solution consist of human insulin

with zink chloride, cholesrol, sodium hydroxide, hydrochloric acid with water .Both of them were used as 10 ml vial of100 IU/ml(as 3.7 mg of human insulin or 40 IU/ kg) .The injection was done by using insulin syringe and for dilution of insulin doses it used beakers of 100 ml .

* **Measurement of insulin dose** : to measure the dose of insulin , it needs to measure the international unit ,which are taken by human body , the dose will be measured in millilitre , in this experiment ,it is used the following formula to measure the dose .

1 IU (International unit) = Kg
100 Kg = 100 IU
1 IU = 1000 gm
X = 20 gm
100 kg = 1 ml

(Ref Buthaina Jasim Al-Anpaki 2004 M.Sc. thesis)

3-Expermintal procedure:

The mice were divided randomly and equally into four major groups of 20 mice each (10 males and10 females) , as low dose , intermediate dose , high(toxic) dose and untreated control group .Treated groups were again subdivided randomly equally into 5 males and 5 females (except the control), treated groups were marked as A,B,C for low ,intermediate and high dose respectively while D group was considered as untreated controls .Treated group " A" , one subgroup of 5 males and 5 females were dosed in equal doses of 0.2 ml of insulin diluted .Solution containing 0.02 IU/ of Actrapid HM while the second group got the same dose of Monotrade HM insulin , both as sub cutanous injection .Group" B" as two subgroups , the first subgroup was injected equally with 0.2 ml of diluted , insulin solution which contained 0.04 IU of 0.2 ml of Actrapid HM insulin ,mice of second sub group were injected exactly with the same dose of Monotrade HM . Group " C" , also of two subgroups , the first subgroup (of 5 males and5 females) were injected sub cutanous of equal dose of 0.2 ml of diluted insulin solution containing 0.06 IU of Actrapid HM, While the second subgroup received exactly the same dose but of Monotrade HM. The forth major group , which was group " D " as untreated control of 10 male & 10 female received 0.2 ml daily sub cutanous of distilled water.

Clinical signs were recorded daily during the six months study period until the end of the study, at the end of the six months period , full post-mortem investigation accomplished .Macroscopic examination was done and the whole internal organs , fixed in 10 % buffered formalin after fixation trimming was done and pieces of about 1 cm were put again in 10 % buffered formalin then slides for microscopical examination were done according to the methods of (13).

- **Body Weights :**Average body weight, was done as for food consumption , average body weights was found and compared with that of untreated control ,that was done weekly for five weeks , in order to know the effect of daily injection of insulin on body weight of animals .Fig(1)Then Body weight graph for insulin treated mice and untreated controls for five successive weeks , which indicate that insulin treated mice started to increase body weights in comparison to control as the experiment proceed .

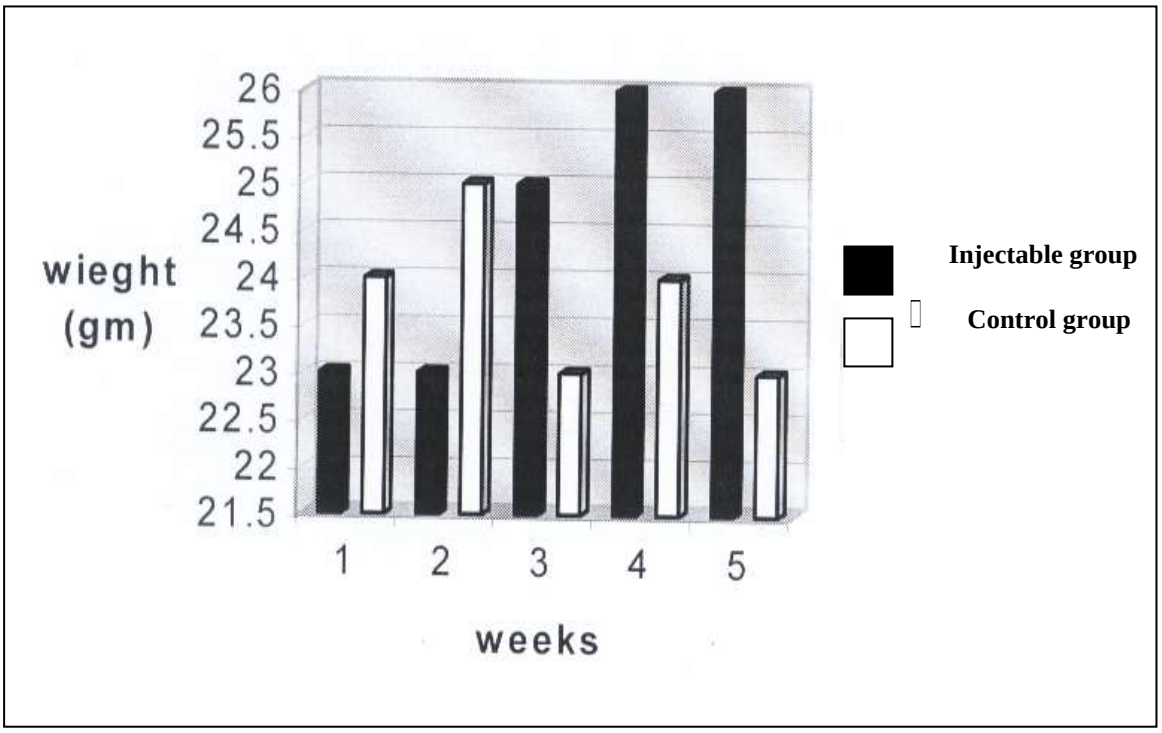


Figure 1

- Food **consumption:** to know food consumption by the experimental animals in comparison with the untreated controls in order to know the effect of insulin on loosing or increase of appetite. Estimation of food was done before and after insulin injection in comparison with controls , that was done weekly for five weeks Fig(2)the present graph showed the affect of daily insulin injection on the average food consumption within five successive weeks .the graph showed decreased of food consumption with continuing treatment , while increase food consumption in untreated controls.

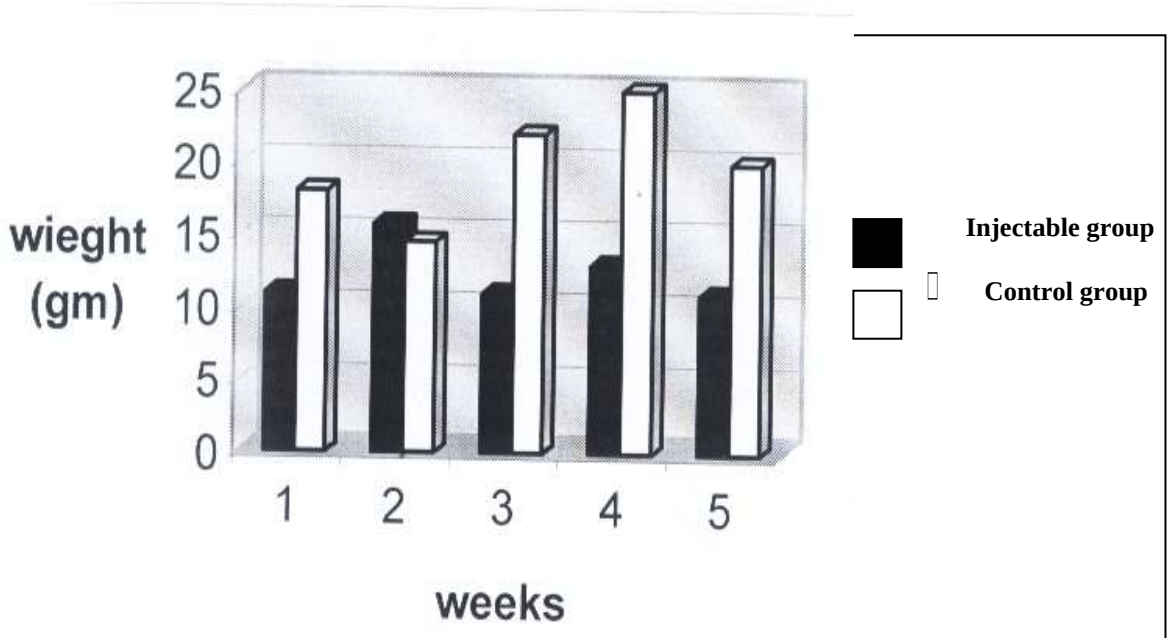


Figure 2

Clinical Findings:

The treated groups showed aggressive behaviour , as injected mice started fighting with each other directly after injection then hours later started to be quite , there was no differences between males and females in the appearance of clinical signs , all treated groups showed to be active healthy with increase of body weight , especially three weeks after injection , while mice started to suffer from signs of hypoglycaemic specially Group A , B (Fig.1) .They were not able to move , Poor condition with generally weak muscle and bones , tremors , general convulsion ,in addition to nervousness and irritability, rapid breath and bradycardia and arch back , then convulsion of all over the body (Fig.2) , Coma and death nearly 100 % in injected mice, especially group B (Intermediate dose group) which they suffered from hypoglycaemic attacks .Other clinical signs were poor condition , they showed piloereaction , lameness in some of the legs , continuous tremor and reduce food consumption in all injected groups, further more , there was loss of elasticity of skin and lipodystrophy ,(Fig 3), in addition to thickened skin and ulceration.

While group B clinically similar to other treated groups but more with 100 % death rate after hypoglycaemic attacks for both males and females ,with clear skin lesions characterized by appearance of pox-like pustules on the abdomen and thigh region , genital and eye lesions (in some cases caused

blindness) mice of group C did not show lesions on eyes , abdomen and back with decrease in rate of death and they were with far less exposure to hypoglycaemic attacks , Untreated control group , they showed evidence of good health conditions and activity with high softness and elasticity of the skin .

Macroscopic Post-mortem findings:

They were about 90 % in similarities in all injected groups those were characterized by presence of layers of fatty tissues especially around the reproductive organs in both males and females, the ovaries, fallopian tube and uterus, epididymus and testis. Also presence of excessive of adipose tissue layers surrounding the kidneys, adrenals, also in subcutaneous regions, especially in females.

Further more, the colour of liver was changing from dark to pale in colour, pancreas was pink to yellow, there was evidence of enlargement of spleen in some of the treated mice (especially those, suffered from repeated attacks of hypoglycaemia). Some of the injected female mice, there was uterine edema, in addition, some treated mice showed sub cutanous edema in the abdominal and thigh regions, directly when the skin opened .

Histopathological Changes:

- **Group A :**

- **Subgroup 1 //** the main histopathological changes characterized by accumulation of large amount of adipose tissue around several visceral organs (Fig-4). Spleen showed lymphoid depletion of the white pulp associated with congestion. While the lung , there was peribronchial lymphoid aggregates and in the interstitium , as well as focal dilatation of alveoli . While the kidneys showed focal areas of dilated cortical tubules. In the liver, there was fatty degeneration with Lymphocytic and mononuclear cellular infiltration in periportal regions and in sinusoids while in brain and spinal cord there were vacculation of neurons in the grey matter .Light microscopy of the ovaries showed presence of corpora lutea and reduced number of mature follicles , with hypertrophy of uterine muscular wall , Testes showed varying stages of spermatogenesis , on some occasions , epididymal tubules appeared empty . There was also adipose tissue infiltration in pancreatic tissue , with focal areas of steatitis , Poor development of islets of langerhans in the pancreas .
- **Subgroup 2 //** this group showed hepatic congestion of sinusoids and periportal region. Pulmonary alveolar congestion , splenic depletion of the white pulp lymphoid tissue and extra modularly haemopoiesis characterized by presence of immature neutrophiles and megakaryocytes(Fig-5).Kidneys showed dilated cortical tubules and enlarged zona fasiculata of the adrenal with vacculation of some cells.Occasionally some mice showed atrophy and poor islets of pancreas with pancreatic infiltration of adipose tissue but to a lesser

extent than in subgroup 1 .In the skin , there was cystic hair follicles . while the brain and spinal cord showed ,vacculation in the nerve fibers of white matter .

- **Group B :**

- **Subgroup 1//** Light microscopy of the lung showed slight lymphocytic infiltration of interstitial tissue , while the kidneys showed cortical aggregates of lymphocytes and dilatation of cortical tubules with glomerular amyloidosis (Fig 6).Splenic perifollicular amyloidosis of white pulp accompanied by lymphoid depletion (Fig-7) while there was lymphoid hyperplasia in white pulp in other mice .Majority of mice seen with depletion of pancreatic islets , hepatic glycogenic infiltration and mononuclear infiltration of uterine glands in some mice with hyperplasia of uterine wall , in addition , other changes were similar as for group A .
- **Subgroup 2//** Fatty degeneration of hypatocytes of some mice , others showed hepatic glycogenic infiltration (Fig – 8). Renal amyloidosis peritubular in cortex and in glomeruli , cortical tubular dilatation and cortical foci of mononuclear cells .Enlargement of zona fasiculata of adrenal cortex . Splenic amyloidosis mainly in perifollicular region , Poor islets of pancreas in some animals , epididymal tubule empty in some treated males (Fig-9) with some missing stages in spermatogenesis in some testis . Ovaries showed presence of corpora lutea , but reduced Graffian follicle ,Uterine glandular hyperplasia and prominent adipose tissue around several of visceral organ .

- **Group C :**

- **Sub group 1 //** Mice in this group showed histopathological changes similar or lesser degree than other previous treated groups. These changes characterized by hepatic vacculation (Mostly as fatty infiltration),glycogenic infiltration in liver , also in the skeletal muscles , mice showed vacuolated islets of the pancreas (Fig-11) .
- **Sub group 2 //** Some .Mice showed renal cortical dilation of tubules as in the other groups, capsular infiltration of mononuclear cells in spleen and depletion of islets in pancreas. There was thickening of uterine wall , Prominent layers of adipose tissue around visceral organs (Fig 12) but to lesser degree than previous treated groups , Changes in ovaries and testes as those reported in previous groups .

Discussion

Insulin considered the main hormone for the pancreas that secreted in response to increase of glucose in blood and also had wide influences on body metabolism therefore any decrease or increase in it's level will cause great damage to tissue and that what was proved by this study .The appearance of nervous signs after insulin injection directly or after hypoglycaemic attacks as it was noted of present study , it appeared that central neurons very sensitive and

depend on glucose as a source of energy (14),therefore when glucose level drop to untolerated level , the mitochondria would be affected as soon as occurrence of hypoglycaemic attack (15) as it was believed that changes in permeability of the inner membrane of mitochondria due to instance of increase calcium level in the cell which result in allowing small molecules to enter mitochondria , which cause disturbances in ATP production , that meant , the hypoglycaemic attacks caused drop in ATP of the brain (16).

Therefore, the nervous signs which were noted in this experiment resulting in degenerative changes in the peripheral and central nerves and that was reported by (17) that hypoglycaemic attacks can cause nervous abnormalities which was more effective in the motor than sensory nerves and could be the cause of hemiplagia as it was noted in the treated mice and those observed signs of the hind legs agreed with what was reported by (18). That nervous abnormalities in diabetes prone to occur in the long nerve fibers and the symptoms quite obvious and clear in legs and feet .One of the macroscopic abnormalities which was showed in the treated mice was loss of elasticity of skin which pointed to the fact that daily injection of insulin and Hyperinsulinemia caused hardening of the cross linking of collagen as it was with hypoglycaemia (19).

The results also showed occurrence of local non specific reaction for both kinds of insulin, for instance allergic reaction and sub. cutaneous odema with the appearance of lipodystrophy and hyperatrophy , because of disturbance in lipid metabolism and due to high level of insulin in blood , as it was reported by (20).The other pathological lesions in the skin of mice , especially group B those could be due to immunosuppression caused by hypoglycaemia , which gave chance for the opportunist viruses and bacteria to cause lesions around and over eyelid , thigh and back .The present formation of several layers of fatty tissue in large quantities in genital region and visceral organs of injected mice in comparison to controls that meant increase level of blood insulin causing changing for the majority of glucose to triglycerides in adipose tissue cells because of activity of enzyme fatty acids synthetase , as it was reported by (21).The mostly yellow colour of liver and Pancreas , that indicated increase level of lipid in the blood lipidaemia , due to disturbance of fat metabolism and formation lipoprotein in the circulating blood which changed triglycerides intracellularly in adipocytes , due to presence of free fatty acids and glycerol, which cause fatty infiltration in visceral organs , and that was agreed with what was reported by (22).

The enlarged spleen especially those mice which suffered repeated hypoglycaemic attacks , could cause compensatory splenic hypertrophy (23).The results of lymphoid atrophy or depletion in the Splenic white pulp , these changes could be due to hypoglycaemic due to insulin level in blood , in

addition to the exhaustion of the bone marrow , as diabetes chronic disease and because of decrease in blood haemopoiesis , that cause activation of Splenic haemopietic stem cells to formed extramedullary haemopoiesis as immature polymorphs and presence of megakaryocytes .Amyloid deposits in spleen of mice from group (B) originated as deposits of gamma globulin probably due to continuous activation of plasma cell as it occurred in chronic infections such as tuberculosis and rheumatic arthritis , which formed amyloid , specially as it was most effective dose which caused increased permeability of the wall of small blood vessels causing deposits of materials in basement membrane , and diffusion of plasma materials as amyloid (8).

The renal histopathological change reveals dilation of renal cortical tubules could be due to the stress caused by hypoglycaemia , as it was observed by (24) , due to thickening of basement membrane , with hyaline deposits as acidic materials , which cause tubular dilation .Renal amyloidosis of group B , especially in glomerular tuft and between cortical tubules , due to activation of endothelial cells and plasma cells and continuous production of globulin materials in the blood (25) The reduced / absence of islets of langerhans , the producer site of insulin in all injected groups , indicate that high level of insulin in the blood , caused negative feed back mechanism causing reduction of beta cells leaving only few of the cell in the islets ,as it was confirmed by (12).the result of disturbance of fat metabolism due to activity of injected insulin , which resulting in hyperlipidaemia , causing fatty infiltration in the cytoplama of Hepatocytes , due to effect of insulin as it was reported by (3) .

The thickening of myometrium in some animals, could be as a response to hormonal endocrine activity as insulin having as effect on steroid hormones (26).the present study also showed thinning of the epidermis and reduced hair follicles ,immature hair follicles , which could be due to reduced blood supply and nervous stimuli to the skin , due to hyperglycaemia because of resistance of cells to insulin (27).

Enlarged zone fasiculata in adrenal of mice injected with insulin, could be due to stress (28).

Fig 1 Mouse (from group B)with hypoglycaemic Attack suffering from coma with ataxia of hind limb, dragging its hind limbs to the back

Fig 2 Two mice(from group C)suffering from hypoglycaemic attacks, one showed arch back , while the other showed signs of turning over.

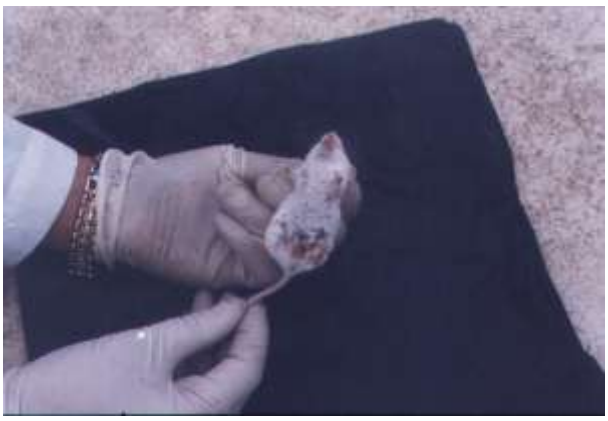


Fig 3 A mouse (from group B)showed lost of the elasticity of skin with associated lipodystrophy

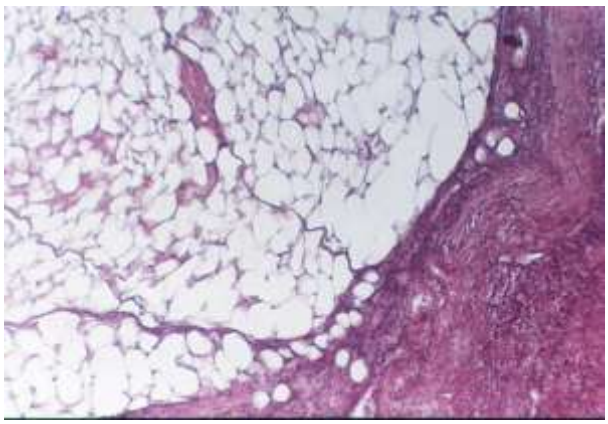


Fig 4 Section of non glandular stomach in a mouse (from group A)show prominent layers of adipose tissue in the serosal and muscular wall (H and E stain X 10)

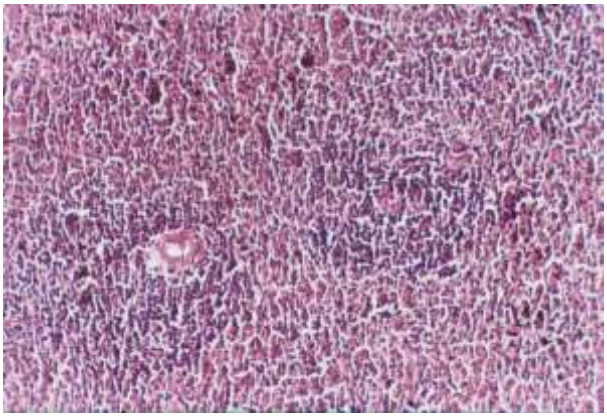


Fig 5 Section of spleen in a mouse (from group A) sub group II , show depletion of lymphoid tissue of white pulp associated with extra meduallry haemopoiesis in red pulp(H and E X 10)

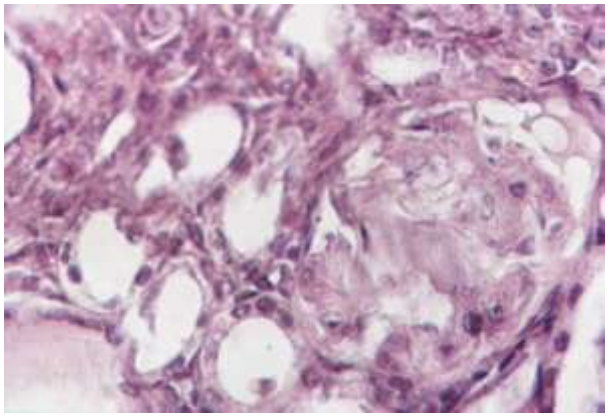


Fig 6 Section of kidney in a mouse (from group B) sub group I showed glomerular deposit of amyloid with cortical tubular dilation (H and E X 40).

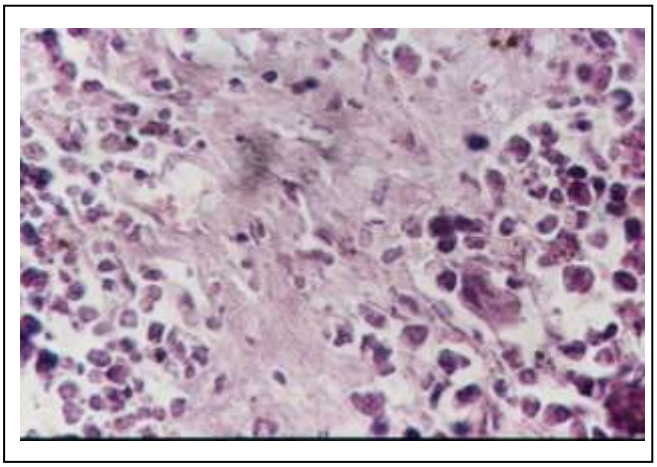


Fig 7 Section of spleen in a mouse (from group B)sub group I showed deposits of amyloid in peri lymphoid follicle of white pulp (H and E stain)

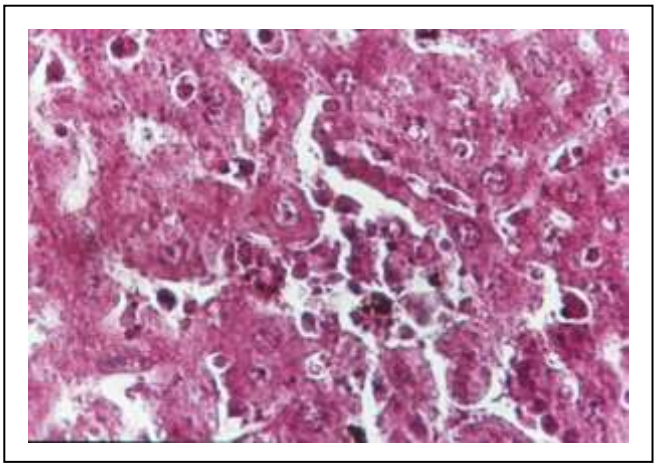


Fig 8 Section of liver in a mouse (from group B)Sub group II , show hepatic cell glycogenic infiltration and mononuclear cells infiltration (PAS, X 40).

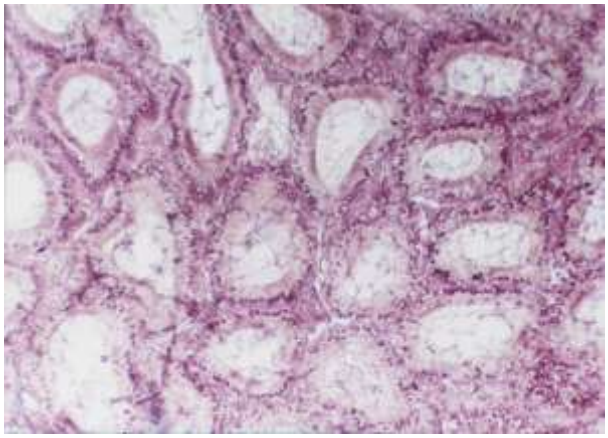


Fig 9 Section of epididymus in a mouse (from group B)Sub group I, showed that tubules empty with no spermatozoa (H and E X 10)

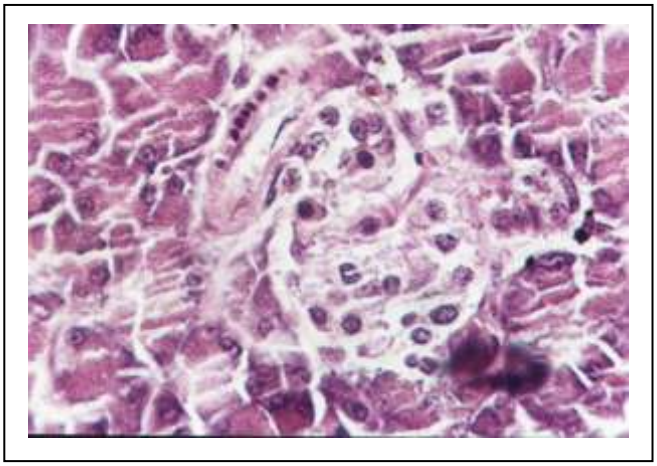
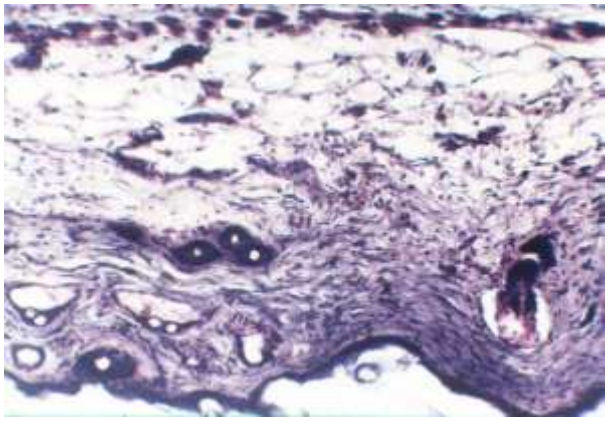
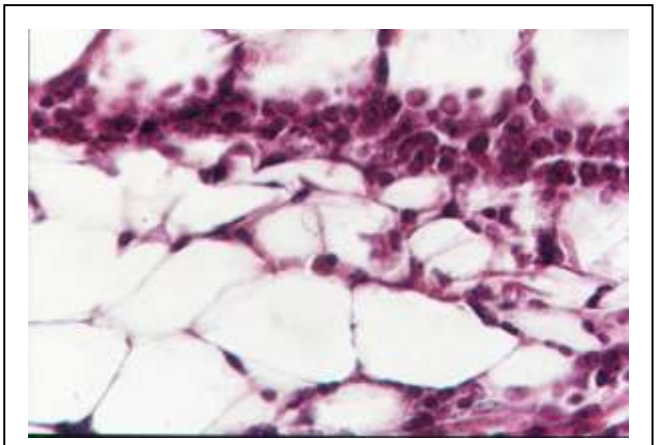


Fig 10 Section of pancreas in a mouse (from group C) subgroup I, showed poor islets, which showed vacculation too (H and E X 40)



Fig(11)Section of skin in a mouse (from group C) Sub group I , showed poor development of hair follicles and prominent dermal adipose tissue (H and E X 40).



Fig(12)Section from visceral organ of adipose tissue in a mouse (from group C)sub group II , showed presence of foci of inflammatory cells ,indicate focal steatitis (H and E X 40).

References

1. Abnergolden, M.N.;Deborath,E. ;Powell,M.D.(1985).Diabetes mellitus . Pathology understanding human disease 2nd ed. Williams and Wilkins Baltimore, London. 404-480.
2. Bilous, R. (2002). Introduction, understanding diabetes 6th ed. BrMed. Ass 1-2.
3. Pickup, J. and Williams, G. (1997).text book of Diabetes .Backwell science LTD .1:1-19.
4. Pinhas, H.O.; Dolan, L.M. and Stanford, D.S. (1996).Increase incident of non insulin dependent diabetes mellitus in children& adolescents .J. pediatric 12:608-615.
5. Shepherd, P.R. and khan, B.B (1999).Mechanism of disease: glugose transport an insulin Action: implications for insulin resistance and diabetes mellitus N.Engl.J.M.341:248-257.
6. Johan, A.; Loraine. And Trevor, B. (1976).hormone assays and their clinical applications .4th ed. Churchill living stone 333-336.
7. Perley, M.J and kipinis, D.M. (1967). The journal of clinical investigation.46:1954.
8. Hoppener, J.O.; Ahren, B.O. and lips, C.J. (2000).Islet amyloid and type 2 diabetes mellitus. engls.med.343:411-419.
9. Osterbly, R.; Gundererson, H.J.; Nybery, G. and Aurell, M. (1987). Advanced diabetes glumerulopathy: quantative structure characterization of non occluded glomeruli-diabites.36:612-619.
10. Larsen, J.R.; Brekke,M.(2001).Glucohemoglobin value over 18 years predict coronary atheromatosis in type 1 diabetes .Diabetologia.44-46.
11. Duck, P. J; Karnes, J. L.; O, Brien, P.; Okazaki, H.; Lais, A. and Engeslstand, J. (1986). The special distribution of fiber loss in diabetic polyneuropathy suggests ischemia .Ann neural.19:440-449.
12. Johan, M. and Kissan, M.D. (1990). Pathologic changes in islets .Anderson pathology9th ed. the C.V.Mosby.comp.Torento.2:1356-1366.
13. Gryer, P .E.; Fisher ,J.N. and Shamoon ,I. T. (1994) .Hypoglycemia Diabetes care .17:734-755.
14. Agardh, C.D. ;Chapman, A.G. ;Perlligrino, D. and Siesjo .B .K(1982) .influence of sever hypoglycemia on mitochondrial & plasma membrane function in brain.J.Neurchm38:662-668.
15. Halestrap, A.P. (1999). The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem.Soc.Symp.66:181-203.
16. Mohseni,S.(2001) .Hypoglycemic neuropathy .Actaneuropathol.102:413-421.
17. Walker,K. (1991).Peripheral neuropathy 2nd ed. Thieme , New york.540-553.
18. Diabetes (2000). http//www.upnaway.com/-poliowa/Diabetes%20-%20me.html.

19. Paterson, K.P .R.; Paice ,B.J. and Lawson ,D.H.(1983).Undesired effects of insulin therapy .Adv. drug react. Ac pois .Rev.2:219-234.
20. Faecett, D.W. (1994).text book of histology. Bloom and Fawceet .Champ man & Hall. New York. London.12ed .176
21. Marchesini, G.; Brizi, M. and Morselli, L.A. (1999). Association of nonalcoholic fatty liver disease with insulin resistance .Amer. J. Med 107:450-455
22. Smith, H .A. ; John, T. C. and Hunt , R.D.(1972). Veterinary Pathology .Lea and Febinger. Philadelphia 4th ed.93.
23. Seyer-hansen, K.;Hansen ,J. and Gundersen ,H.J.(1980).Renal hypertrophy in experiment diabetes .diabetologia .18:501-505.
24. Osborne, C.A (1968).Renal amyloidosis in the dog .J.Amer.Vet.Asso.153:669-668.
25. Joan, F. and Zliva, A. (1975). Clinical chemistry in diagnosis and treatment 2nd ed.london.185-187.
26. Luna, L.G. (1968). Manual of histology staining of the armed forces institute of pathology .3rd.U.S.A. Megrew Hill Bock.
27. Al-Anapaki, Buthaina .J.Jwaad. (2004).experimental pathological study of effect of variable insulin doses in rat and mice .M.Sc. thesis.
28. Majeed, S.K. Personal observation2006.