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RESEARCH ARTICLE

Evaluation of Combined Effects of Linagliptin and Metformin on Fertility and Toxicity Parameters in Male Wistar Rats

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ABSTRACT

Monotherapy failure within short period of time is commonin Type-2 diabetes patients and becomes a challenge to healthcare providers around the world. A newer trend in current clinical practice is to prescribe a combination of metformin with incretin-based therapies. One such combination is metformin along with linagliptin for type 2 diabetes and proved to be promising for patients unable to achieve HbA_{1c} (glycated haemoglobin) targets with traditional therapy. The aim of this study was to investigate effect whether co-administration of linagliptin and metformin drugs produces any unexpected or cumulative toxic and/or adverse effects on male fertility including toxicity profile as compared to adverse effect reported by individual drugs. Three treatment groups having 6 male rats in each group were administered linagliptin at dose rate of 0.5, 1 and 2 mg/kg combined with administration of metformin at doe rate of 205, 410 and 820 mg/kg, control rats were administered with filtered water as vehicle for consecutive 28 days. All the male rats were normal throughout experimental period and no adverse changes were observed in hematological, biochemical parameters except alteration in cholesterol and creatinine at mid and high doses. . Organ weights remained unaffected except lower absolute weights of spleen, thymus and lungs at high doses. Sperm analyses such as motility, morphology and epididymal sperm count did not revealed any adverse changes between different treatment and control groups. No treatment related adverse effects were noticed in weight of testes, epididymis and other accessory reproductive organs up to high dose. No treatment related gross pathological effects were noticed however, minimal to mild hepatocellular hypertrophy in liver and dilated tubules in kidney were observed microscopically in all the animals treated at high dose group. In conclusion, daily co-administration of linagliptin and metformin by oral route up to the dose level of 2mg/kg and 820 mg/kg, respectively for 28 days did not impair the fertility in male Wistar rats however, based on significant alteration in body weight and pathological findings, the No-Observed-Adverse-Effect-Level (NOAEL) for the linagliptin and metformin combination was considered to be 1mg/kg and 410 mg/kg under these study conditions.

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INTRODUCTION

Diabetes mellitus is one of the most common chronic diseases in nearly all countries and continues to increase in numbers and significance, as economic development and urbanization lead to changing lifestyles characterized by reduced physical activity and increased obesity (Whiting *et al*., 2011).

Over the last 15 years several global estimates of the prevalence of diabetes have been estimated that there would be 300 million adults with diabetes in 2025 (King *et al.,* 1998); in 2004 WHO estimated 171 million for 2000 and 366 million by 2030(Wild *et al.,* 2004); and previous editions of the IDF Diabetes Atlas have estimated the global prevalence to be 285 million in 2010 (IDF, *2nd ed.,* 2009). The current prediction is that the

world wide prevalence of diabetes would be higher than 552 million by the year 2030. So, diabetes continues to be a major public health challenge around the globe.

Diabetes is a syndrome of impaired carbohydrate, fat and protein metabolism caused by either lack of insulin secretion or decreased sensitivity of the tissues to insulin. The incidence of type 2 diabetes is increasing worldwide as a consequence of changing lifestyle and prevalence of obesity and metabolic syndrome. Clinically higher levels of blood glucose, caused by decreased insulin secretion, insulin resistance or both, characterize type 2 diabetes. In diabetic patients, it is important to control the elevated blood glucose levels, both for the prevention or delaying the progression of diabetic complications.

Current guidelines by the American Diabetes Association (ADA), (Nathan *et al.,* 2009) the American Association of Clinical Endocrinologists (AACE), (Rodbard *et al.,* 2007)the International Diabetes Federation (IDF), the UK National Institute for Clinical Excellence (NICE), and the Canadian Diabetes Association (CDA) for the pharmacologic management of type 2 diabetes recommend lifestyle modifications (weight reduction, dietary adjustments and physical exercise) followed by initial monotherapy and, subsequently, a stepwise intensification of therapy if glycemic control is inadequate. Patients with excessive HbA_{1c} levels after initiating metformin should introduce additional antihyperglycemic agents into their treatment regimens until target glycemia (whether defined as <6.5% or <7%) is achieved (Nathan *et al.,* 2009, Rodbard*et al.* 2007).

Considering the therapeutic potential of combination therapy for the safe and effective treatment of type 2 diabetes, a world-wide effort for developing a fixed dose combination is directed towards the discovery of new treatments that alleviate this medical problem. As part of this effort, Linagliptin (DPP-4 inhibitors) in combination with Metformin is preferred when diet & exercise plus Metformin alone do not provide adequate glycemic control. These drugs together target a number of key defects in the diabetes pathology, so may help achieve a tighter control of the patient's blood glucose levels. Most patients with type 2 diabetes are treated with Metformin as monotherapy, as well as with other hypoglycemic drugs, which is capable of maintaining a good metabolic control for a limited period of time. For this reason, most type 2 diabetic patients, after a few years from diagnosis, require combined treatments of drugs having complimentary mechanism of action in order to reach therapeutic goals.

A general practitioner in such cases would prescribe a combination based on current treatment paradigms, but such combinations are not well studied in pre-clinical background. Given the known biological action of the incretin therapies, they would appear to be particularly attractive agents to be used in combination to achieve HbA_{1c} goals. Linagliptin is the fifth Dipeptidyl-peptidase-IV inhibitor inhibitor application submitted for FDA review for approval to treat type 2 diabetes (Paul C. Brown, 2010). Most DPP-4 inhibitors are given with a standard dose once daily, fixed dose combinationswith metformin are available. Adding Linagliptin to an insulin sensitizer such as Metformin addresses the three key defects of type 2 diabetes: insulin resistance, β-cell dysfunction and α-cell dysfunction. So this combination therapy is a promising choice to achieve glycaemic goals as per current guideline. Fixed dose combination of DPP-4 inhibitor with metformin are feasible and in practice patient adherence has been good to the fixed dose combinations. Therefore, this drug class has been perceived as an important addition to the oral antidiabetic treatment options. DPP-4 inhibitors are an oral drug class with a mode of action based on incretin physiology

In view of literature search of both drugs, we proposed to find out whether co-administration of representative drugs produces any unexpected or cumulative toxic and/or adverse effects as compared to adverse effect reported by individual drugs.

This study hopes to shed light on the major toxic effects of Linagliptin and Metformin by co-administration in healthy male wistar rats, with particular emphasis on target organ of toxicity and the potential adverse effect on fertility as a result of 28 days repeated oral exposure of the drug to experimental animals.

MATERIALS AND METHODS

Test Items: Linagliptine (Batch No.LIA-API-1-146) and Metformin (Batch No. MT03430311) were made available from Cadila Healthcare Limited, Ahmedabad, India.

Experimental Animals: Animals were made available from Animal Research Facility, Zydus Research Centre, Ahmedabad. The study was conducted under approval of IAEC(protocol No. ZRC/TOX/BP/011/08-2K12) in healthy young adult Wistar rats of both genders. Rats in group of three were housed individually ventilated cages. During cohabitation, single male was housed with two proven females. All mated females were housed individually. Temperature and humidity was maintained between 21-29 °C and 30-70%, respectively with12 hours light, 12 hours dark cycle in the experimental room.

Experimental Design

Dose selection: The doses were selected based on the MRHD and published literature of individual drug preclinical data. The dose levels selected for the study were Linagliptin : 0.5, 1 and 2 mg/kg which is 1 X, 2 X and 4 X and Metformin : 205, 410 and 820 mg/kg which is 1 X, 2 X and 4 X of human equivalent rat dose.

Male Fertility Study: Male rats were randomized into three treatment groups and one control group having 6 animals per group based on body weight. Proven females rats used for mating purpose in this study were allocated to different groups based on the body weight. The metformin and linagliptin in combination at the dose level of 0.5+205, 1+410 and 2+820 mg/kg body weight was administered to male rats by oral gavage for a period of 14 days prior to mating and thereafter treatment were continued during cohabitation with untreated females up to their scheduled terminal sacrifice. Females were sacrificed on presumed gestation day 14 to evaluate fertility. Concurrent control group animals were administered with vehicle (filtered water) alone.

Parameters studied:

Gross & histopathological examination: At the end of experiment all the animals were euthanized with CO_2 asphyxation and detailed necropsy examination was performed. Organs were collected for weighing and subjected to histopathological examination on H&E stained slides.

Sperm Evaluation was carried out for all groups at the scheduled terminal sacrifice. One of the epididymis was used for analysis of sperm motility, sperm count and sperm morphology. **Uterine observations:** The mated untreated females were sacrificed at mid gestation period i.e. on presumed gestation day 14 and uteri was examined for pregnancy status and fertility index was calculated. The gravid uterus along with cervix and ovaries was weighed and were observed for number of corpora lutea, total number of implantation sites, number of live and dead conceptuses and number of resorptions.

The following formulas were used for calculating reproductive indices (Hayes, A.W., 2000).

Male Fertility Index (%) = Number of males impregnating a female/ Number of males cohabitated X 100

Female Fertility Index (%) = Number of pregnant females/ Number of females cohabitated X 100

Body Weight Gain (g) = Current body weight (g) –Previous body weight (g)

Maternal Corrected Body weight (g) = Terminal body weight (g) – Uterus weight (g)

Relative Organ to Brain Weight (%) = Organ weight (g)/ Brain weight (g) X 100

Sperm Motility (%) = Total number of sperms counted – number of non-motile sperms/ Total number of sperms counted X 100

% Normal Sperms = Total number of sperms observed – number of abnormal sperms/ Total number of sperms observed X 100

% Abnormal Sperms = Number of abnormal sperms/ Total number of sperms observed X 100

Sperm per cauda Epdidiymis (millions/mL) = Total sperms counted X dilution volume X hemocytometer factor/ Volume of counting chamber X 10^6

Volume of counting Chamber = Area counted X Depth of fluid

Area counted = 1/25 X5 = 1/5 Sq.mm

Depth of Fluid = 0.1 mm

Hemocytometer factor = 1000

Sperm per gram cauda Epdidiymis (millions/g tissue) = Sperms counted per cauda (millions /mL)/ Weight of Cauda (g)

Total number of Resorptions = No. of early resorptions + No. of late resorption

Statistical analysis

The data are represented as mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism Version *6.00*. ANOVA (Analysis of Variance) was used for the comparison of different dosage groups with the control group for body weight, body weight change, organ weight, sperm motility and sperm count. Non-parametric ANOVA test such as Kruskal-Wallis test was employed for analysis of live and dead implants, corpora lutea count, no. of implantations and resorptions. Unpaired Student's t-test was used to analyze sperm morphology data.

RESULTS AND DISCUSSION

General clinical signs

All male and female animals were survived till the end of treatment period. Treatment with different doses of Linagliptin and Metforminas concominant administration did not show any clinical sign during the study period. Male animals in all treatment groups were found normal. All females mated with treated male rats from different groups were found normal throughout the gestation period. No clinical signs of toxicological significance were noticed including ophthalmogcal examination. As reported by earlier researcher (Qualie, et al., 2010), toxicity study of longer duration (13 weeks) with metformin alone in Crl: CD (SD) ratsmay result into moribundity/mortality and clinical signs of toxicity in the animals.

Body weight

Treatment associated declining trend in body weight was noticed in male animals, which was found to be statistically significant at high dose during week-3 and 4 when compared with concurrent control group. This observation in this study was in agreement with reduction in body weight gain reported in Zuker fa/fa rat when Metformin administered in combination with Dipeptidyl Peptidase IV Inhibitor (Yasuda, N., *et al,* 2004). Mean body weight change from low and high dose revealed statistically significance during experimental period as compared to control group rats. There was no change in mean body weights of females through out the experiment

Clinical pathology

Terminal haematological analysis of male animals did not reveal any treatment dependent changes compared to control group. Serum biochemical analysis of male animals showed higher cholesterol level (group II), lower creatinine levels (group IV) compared to concurrent control group. All these changes were considered to be incidental as they did not reveal dose dependent trend and were within normal historical control range. No treatment related variations noticed during urinalysis in male rats up to high dose. Urine was clear yellow to pale yellow color at all dose levels. No adverse changes were evident in specific gravity and other physical, chemical and microscopic examination.

Organs weight and feed consumption

Organ weights from all different treatment groups remained unaffected except absolute weights of spleen, thymus and lungs were low at high dose while absolute spleen weight was also reduced in low dose group male rats. Males treated with high dose exhibited higher relative weight (relative to body weight) of heart, liver, brain and testes when compared with respective control group. Brain relative weight was also altered in mid dose group male rats. Further, males at high dose showed statistically altered relative weight (relative to absolute brain weight) of heart, thymus, lung and spleen when compared with respective

Table 1: Summary of body weight (g) – male (Mean ± SD, n=6)

Dose Frequency	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
Day-1	191.33 ±16.13	189.03 ±12.96	189.55 ±16.06	191.08 ±15.12
Week-1	219.60 ±14.92	212.48 ±12.58	211.90 ±17.28	212.03 ±17.13
Week-2	247.38 ±15.85	236.67 ±12.31	231.55 ±17.18	231.25 ±19.11
Week-3	269.82 ±17.05	251.45 ±13.03	249.83 ±19.83	240.70 ±16.97*
Week-4	288.23 ±19.38	272.43 ±15.88	265.92 ± 20.85	249.65 ±17.90**

*: Significant at 5% level Vs Control (P<0.05), **: Significant at 1% level Vs Control (P<0.01).

Table 2: Summary of group mean body weight change (g) -male (Mean ± SD, n=6)

Dose Frequency	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
Week 1	28.27 ± 2.96	23.45 ± 4.20	22.35 ± 3.50*	20.95 ± 4.60*
Week 2	27.78 ± 4.57	24.18 ± 4.73	19.65 ± 3.65*	19.22 ± 4.38**
Week 3	22.43 ± 1.35	14.78 ± 2.63*	18.28 ± 8.09	9.45 ± 3.56***
Week 4	18.42 ± 4.01	20.98 ± 6.48	16.08 ± 3.67	8.95 ± 3.72**

* Significant at 5% level vs Control (P<0.05), ** Significant at 1% level vs Control (P<0.01), *** Significant at 0.1% level vs Control (P<0.001).

Table 3: Summary of group mean body weight (g) (mean ± sd, n=6) during gestation – female

Gestation Day	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
0	193.96 ± 12.43	191.86 ±11.31	193.06 ±11.43	193.84 ±10.92
7	214.40 ±14.57	212.78 ±14.92	213.05 ±15.58	214.78 ±12.06
14	241.46 ±16.04	239.50 ±16.46	241.60 ±16.59	237.69 ±16.30

Table 4: Summary of haematological estimations (Mean ± SD, n=6) male

Analytes	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
WBC (10^3µL)	10.26 ± 1.46	7.70 ±1.54	8.23 ±1.91	10.82 ±2.89
RBC (10^6µL)	7.62 ±0.31	7.77 ±0.36	7.79 ±0.36	7.99 ±0.33
HGB (g/dL)	14.40 ±0.50	14.63 ±0.30	14.40 ±0.54	14.58 ±0.37
HCT (%)	45.73 ±1.76	46.07 ±1.24	45.62 ±1.65	46.07 ± 1.17
MCV (fL)	60.07 ±1.25	59.37 ±2.16	58.65 ±2.19	57.70 ±1.69
MCH (pg)	18.93 ±0.32	18.87 ±0.69	18.52 ±0.78	18.23 ±0.54
MCHC (g/dL)	31.52 ±0.34	31.82 ±0.43	31.58 ±0.19	31.63 ±0.37
PLT (10^3µL)	726.33 ±45.14	754.67 ±109.90	709.00 ±42.00	774.67 ±63.16
NEU (10^3µL)	1.442 ±0.276	1.065 ±0.145	1.060 ±0.305	1.161 ±0.345
LYMP (10^3µL)	8.430 ±1.288	6.258 ±1.689	6.618 ±1.480	9.380 ±2.695
MONO (10^3µL)	0.220 ±0.124	0.213 ±0.165	0.344 ±0.113	0.100 ±0.062
EOS (10^3µL)	0.084 ±0.036	0.097 ±0.039	0.101 ±0.054	0.089 ±0.033
BASO (10^3µL)	0.082 ±0.034	0.076 ±0.019	0.095 ±0.032	0.079 ±0.054

Table 5: summary of clinical biochemical estimations (Mean ± SD, n=6) – male

Analyses	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
GLU (mg/dL)	102.83 ±6.62	93.90 ±13.41	90.60 ±19.96	111.50 ±18.57
TG (mg/dL)	102.62 ±38.94	117.48 ±41.58	87.70 ±22.69	92.82 ±20.32
CHO (mg/dL)	52.78 ±9.81	71.00 ± 11.60*	58.87 ±7.50	59.83 ±10.76
AST (U/L)	104.93 ±15.01	111.07 ±20.96	104.82 ±15.48	124.85 ±14.36
ALT (U/L)	28.95 ±2.22	30.72 ±5.79	28.43 ±4.98	32.47 ±5.18
ALP (U/L)	110.27 ±22.56	125.03 ±11.47	124.45 ±31.75	117.63 ±25.40
TBL (mg/dL)	0.22 ±0.02	0.21 ±0.01	0.22 ±0.02	0.21 ±0.02
TP (g/dL)	6.00 ±0.23	5.93 ±0.37	5.97 ±0.16	5.88 ±0.25
ALB (g/dL)	3.70 ±0.09	3.70 ±0.20	3.70 ±0.09	3.63 ±0.12
GLB (g/dL)	2.50 ±0.64	2.23 ±0.18	2.27 ±0.12	2.25 ±0.15
UREA (mg/dL)	30.63 ±6.31	31.72 ±4.49	29.65 ±3.61	31.65 ±4.71
CRE (mg/dL)	0.65 ±0.05	0.62 ±0.07	0.56 ±0.03	0.46 ± 0.21*
Ca (mg/dL)	10.55 ±0.22	10.95 ±0.38	10.93 ±0.23	10.82 ±0.31
Ph (mg/dL)	10.57 ±0.31	11.13 ±0.29	10.83 ±0.32	10.57 ±0.12
Na (mmol/L)	145.73 ±0.31	144.83 ±0.91	144.90 ±1.78	145.22 ±5.14
K (mmol/L)	3.77 ±0.17	3.90 ±0.33	3.85 ±0.16	3.91 ±0.33
Cl(mmol/L)	30.63 ±6.31	31.72 ±4.49	29.65 ±3.61	31.65 ±4.71
A:G	1.538 ±0.271	1.658 ±0.052	1.637 ±0.090	1.618 ±0.083

Key: * = Significant at 5% level Vs Control (P<0.05).

Table 6: Summary of absolute organ weights (g) (Mean ± SD, n=6) male

Organs	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
Adrenals	0.054 ± 0.004	0.057 ±0.007	0.058 ±0.009	0.054 ±0.011
Heart	0.838 ±0.070	0.823 ±0.080	0.852 ±0.126	0.942 ±0.077
Kidneys	1.692 ±0.156	1.653 ±0.242	1.573 ±0.156	1.552 ±0.168
Liver	8.485 ±0.748	7.638 ±0.789	8.209 ±1.193	8.145 ±0.820
Spleen	0.604 ±0.061	0.499 ±0.039**	0.529 ±0.039	0.466 ± 0.068***
Testes	3.171 ±0.268	3.122 ±0.148	3.188 ±0.266	3.031 ±0.320
Epididymides	1.099 ±0.132	1.107 ±0.138	1.094 ±0.146	0.991 ±0.081
Thymus	0.429 ±0.102	0.371 ±0.106	0.354 ±0.115	0.278 ± 0.042*
Lung	1.389 ±0.107	1.271 ±0.190	1.403 ±0.147	1.122 ± 0.029**
Brain	1.891 ±0.141	1.908 ±0.034	1.996 ±0.077	1.830 ±0.079
Prostate/Sv-cog	1.676 ±0.213	1.361 ±0.305	1.488 ± 0.256	1.399 ±0.153

Key: * = Significant at 5% level Vs Control (P<0.05), ** = Significant at 1% level Vs Control (p<0.01), *** = Significant at 0.1% level Vs Control (P<0.001)

Table 7: Summary of relative organ weights (%)(Mean ± SD, n=6) – male (Organ - Body Weight Ratio)

Group treatment	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
terminal wt. wtweights (g)	274.93 ±21.02	254.92 ±14.77	248.22 ± 22.12	231.68 ±20.95 20.95**
adrenals	0.020 ±0.002	0.022 ±0.002	0.023 ±0.004	0.024 ±0.004
heart	0.305 ±0.018	0.322 ±0.015	0.343 ± 0.035	0.409 ±0.048****
kidneys	0.617 ±0.054	0.651 ±0.109	0.634 ± 0.032	0.669 ±0.019
liver	3.092 ±0.249	2.990 ±0.150	3.297 ± 0.264	3.516 ±0.147**
spleen	0.221 ±0.026	0.196 ±0.010	0.214 ± 0.013	0.204 ±0.042
testes	1.161 ±0.157	1.227 ±0.066	1.286 ± 0.070	1.309 ±0.070*
epididymides	0.403 ±0.061	0.433 ±0.035	0.443 ± 0.064	0.430 ±0.042
Thymus	0.156 ±0.032	0.145 ±0.041	0.142 ±0.039	0.121 ±0.022
Lung	0.508 ±0.053	0.497 ±0.051	0.567 ±0.058	0.487 ±0.041
Brain	0.692 ±0.083	0.751 ±0.048	0.808 ± 0.055**	0.793 ±0.048*
Prostate/Sv-cog	0.613 ±0.089	0.531 ±0.098	0.598 ±0.079	0.604 ±0.033

Key: * = Significant at 5% level Vs Control (P<0.05), ** = Significant at 1% level Vs Control (P<0.01), *** = Significant at 0.1% level Vs Control (P<0.001), SV-COG = Seminal vesicle with coagulating gland.

Table 8: summary of relative organ weights (%) – male (Organ - Brain Weight Ratio)

Organs	Group I Vehicle Control (0 mg/kg)	Group II Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Group III Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Group IV Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
Adrenals	2.852 ±0.384	2.978 ±0.365	2.918 ±0.453	2.977 ±0.604
Heart	44.439 ±4.046	43.143 ±4.530	42.595 ±5.422	51.553 ±4.892*
Kidneys	90.127 ±12.517	86.617 ±12.679	78.773 ±6.444	84.652 ±6.201
Liver	450.706 ±49.529	400.520 ±44.080	410.467 ±51.564	445.104 ±38.009
Spleen	32.204 ±5.173	26.122 ±1.983*	26.507 ±1.823*	25.628 ±4.779*
Testes	168.779 ±22.212	163.710 ±9.439	159.499 ±8.175	165.666 ±15.333
Epididymides	58.409 ±8.036	58.056 ±7.879	54.744 ±6.310	54.245 ±4.683
Thymus	22.683 ±4.777	19.504 ±5.759	17.728 ±5.726	15.170 ±2.212*
Lung	73.814 ±7.801	66.627 ±10.309	70.288 ±6.697	61.449 ±3.503*
Prostate/Sv-cog	88.984 ±12.128	71.391 ±16.200	74.377 ±11.362	76.401 ±6.767

* = Significant at 5% level Vs Control (P<0.05), SV-COG = Seminal vesicle with coagulating gland

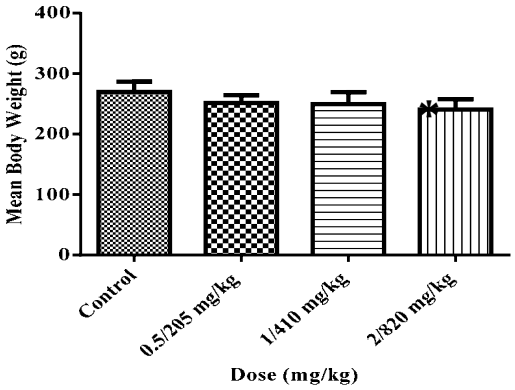
control group. Relative organ weight of spleen was also altered in low and mid dose group male rats. Feed consumption of male and females rats from different treatment group was comparable throughout experiment period and did not reveal any statistical or biological significance. It was reported (Yasuda, N., *et al,* 2004) that Metformin treatment in Zucker fa/fa rats reduce the food intake and body weight gain in combination with Dipeptidyl Peptidase IV Inhibitor.

Gross & histopathology

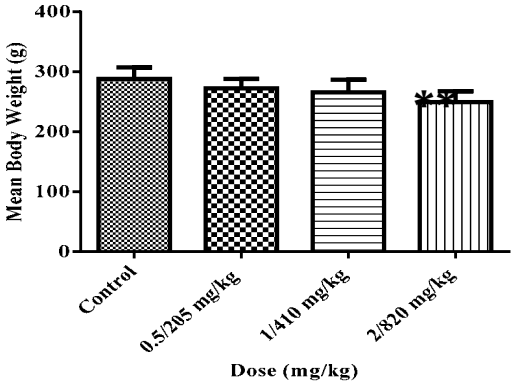
Gross pathological examination of both male and female animals did not reveal any abnormalities attributed to treatment of combination drugs up to the high dose. Microscopically, minimal to mild hepatocellular hypertrophy in liver and dilated tubules in kidney was observed in all the animals at group IV and was considered as test item-related findings. Additionally, stress related findings like minimal to mild lymphoid depletion in thymus and spleen in few animals of group IV was observed. There were no any histological findings in remaining groups. Increased incidence of minimal necrosis with minimal to slight inflammation of the parotid salivary gland for males given 1200 mg/kg/day reported in 13 weeks study in rats (Qualie, *et al*., 2010).

Sperm evaluation

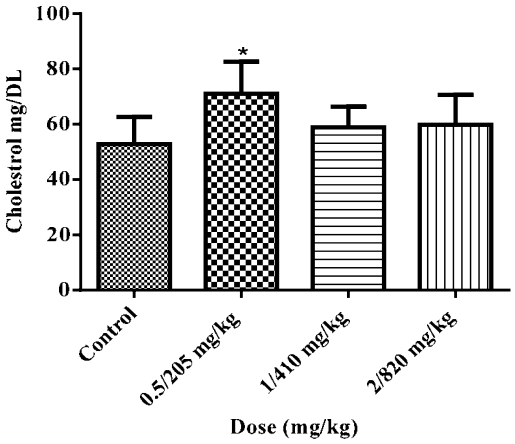
No test item related effects were noticed in sperm motility up to 2.0+820 mg/kg compared to control groups. Total number of epididymal sperm count in Linagliptin and



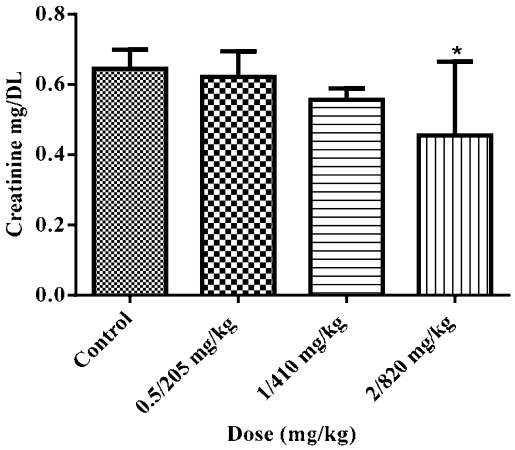
Graph-1: Body weight (g) week 3 – male



Graph 2: Body weight (g) week 4 – male



Graph: Cholesterol – male



Graph: Creatinine - male

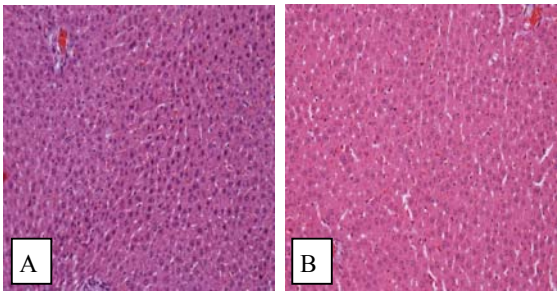


Fig. 1a: Control Male-Liver (20X) Showing Normal appearance. **B:** High Dose Male-Liver (20 X) Showing Hepatocellular Hypertrophy

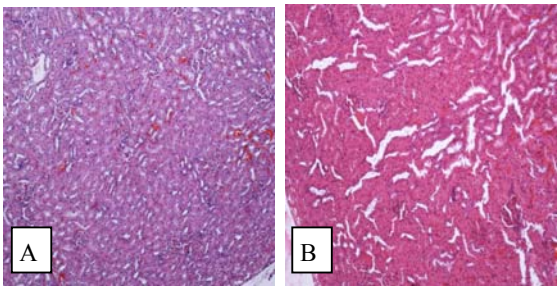


Fig. 2a: Control Male-Kidney (10 X) Showing normal appearance; **b:** High Dose Male-Kidney (10 X) showing dilated renal tubules

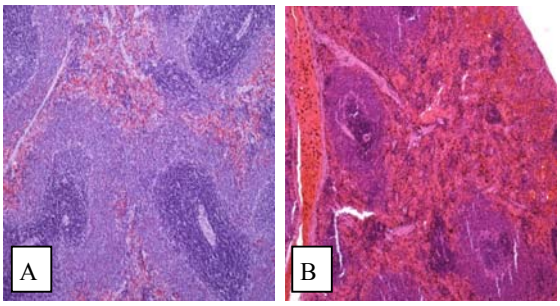


Fig. 3a: Control Male-Spleen (10 X) showing normal appearance; **b:** High Dose Male-Spleen (10 X) showing lymphoid depletion

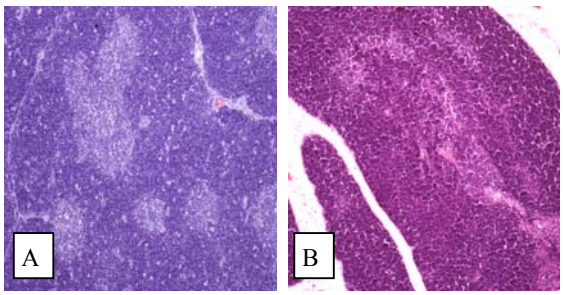


Fig. 4a: Control Male-Thymus (10 X) showing normal appearance; **b:** High Dose Male-Thymus (10 X) showing lymphoid depletion

Metformin treated rats revealed comparable count to the concurrent control groups. No treatment related effects were noticed in sperm morphology at the high dose and the changes noticed in both control and treated animals were common findings that are routinely seen in normal adult rats.

Table 9: Summary of sperm analyses (Mean ± SD, n=6)

Parameter	Group I	Group II	Group III	Group IV
	Vehicle Control (0 mg/kg)	Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
% Motile Sperm	83.87 ±6.537	88.67 ±1.106	88.28 ±2.317	87.57 ±1.859
% Non Motile Sperm	16.13 ±6.537	10.77 ±1.586	11.72 ±2.317	1 2.43 ±1.859
Sperm Count (millions/ml)	29.17 ±5.46	35.50 ±9.07	31.67 ±6.31	32.17 ±6.97
Sperm Count (millions/g cauda)	534.11 ±111.93	639.42 ±131.91	548.43 ±127.24	570.51 ±98.00
% Normal Sperms	96.67 ±0.41	Not evaluated as there was no changes at 2+820 mg/kg		97.00 ±0.63
% Abnormal Sperms	3.33 ±0.41			3.00 ±0.63

Table 10: Summary of uterine data (Mean ± SD, n=12)

Observations	Group I	Group II	Group III	Group IV
	Vehicle Control (0 mg/kg)	Linagliptin (0.5 mg/kg) + Metformin (205 mg/kg).	Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
14^{th} day Corrected B. wt. (g)	232.22 ±16.26	231.63 ±15.39	232.68 ±16.13	230.56 ±16.55
Uterine Weight (g)	9.24 ±1.14	7.88 ±2.71	8.92 ±1.4	7.14 ± 2.57*
Relative Uterine Weight (%)	3.85 ±0.59	3.27 ±1.04	3.7 ±0.52	3.02 ±1.12
No. of Corpora Lutea	11.83 ±1.03	11.25 ±4.31	11.83 ±0.94	9.75 ±4.41
Total No. of Implants	11.5 ±1	9.75 ±3.57	11.17 ±1.34	8.83 ±4.13
No. of Live Conceptuses	10.67 ±1.56	9.42 ±3.55	10.75 ±1.36	7.75 ±4.09
Total No. of Resorptions (Early + Late)	0.83 ±1.7	0.33 ±0.49	0.42 ±0.67	1.08 ±1.44
Implantation Index (%)	97.3 ±4.05	88.0 ±12.14	94.5 ±10.14	88.2 ±16.06

* = Significant at 5% level Vs Control (P<0.05).

Table 11: summary of fertility indices

Group	Group I	Group II	Group III	Group IV
Treatment	Vehicle Control (0 mg/kg)	Linagliptin (0.5 mg/ kg) + Metformin (205 mg/kg).	Linagliptin (1 mg/kg) + Metformin (410 mg/kg)	Linagliptin (2 mg/kg) + Metformin (820 mg/kg)
Total Number of Males per group	6	6	6	6
No. of Males used for Impregnating Untreated Females	6	6	6	6
Total No. of Untreated Females Used for Cohabitation	12	12	12	12
No. of Females Mated	12	12	12	12
No. of Females Pregnant at Sacrifice	12	11	12	11
Male Fertility Index (%)	100.0	100.0	100.0	100.0
Female Fertility Index (%)	100.0	91.67	100.0	91.67

Uterine data

Male rats treated with Linagliptin and Metformin up to 2.0+820.0 mg/kg mated with untreated proven females did not reveal any adverse effects on uterine parameters such astotal number of corpora lutea, total number of implants, number of live and dead conceptuses and number of resorptions, however gravid uterine weight was slightly reduced statistically in high dose group female rats. Relative uterus weight and corrected maternal body weight were comparable with the concurrent control group.

Fertility indices

No treatment related changes was noticed up to 2.0+820.0 mg/kg. The male fertility index was 100% in all the groups and the female fertility index was 100.0, 91.67, 100.0 and 91.67% in control, low (0.5=205.0 mg/kg), mid (1.0+410.0 mg/kg) and high (2.0+820.0 mg/kg) dose groups respectively.

Although metformin has been marketed for many years, there are only limited non-clinical data available in published literature. Therefore, the general and reproductive toxicity of metformin was investigated in rat studies. Repeat-dose toxicity studies and reproduction toxicity studies were conducted with the fixed dose combination linagliptin/metformin (Assessment report, Jentadueto, EMEA, 2011) and reported that such combination of linagliptin and metformin does not affected the general reproductive performance and fertility indices.

Conclusion

Daily oral administration of linagliptin and metformin for 28 days at the dose levels of 0.5+205, 1+410 and 2+820 mg/kg, respectively did not affect the survival of male Wistar rats. All male rats were normal throughout experimental period in this study. In conclusion, daily administration of Linagliptin and metformin by oral route up to the dose level of 2+820 mg/kg for four consecutive weeks did not impair the fertility in male Wistar rats however based on significant alteration in body weight and pathological findings, the No Observed Adverse Effect Level (NOAEL) was considered to be 1+410 mg/kg (Linagliptin + Metformin) under these study conditions.

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