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Research Article

A Study on Preclinical Safety and Efficacy of MCP I Conjugated Gold Nanoparticles based oral contraceptive using Biomarkers of Reproductive Tissues and Blood Serum of Male Albino Wistar Rats

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ABSTRACT

Characterized MCP I fraction of *Carica papaya* seeds is being considered a potential contraceptive agent. Utilization of gold nanoparticles as a carrier molecule enhanced its targeted bioavailability, biocompatibility, in vitro stability against blood serum and pH gradient. In this study, preclinical safety and efficacy were assessed employing concerned biomarkers of blood serum and excised reproductive tissues for further approval of this nanoformulation. Experimental groups A, B and C were consists of vehicle control, orally treated with MCP I (50 mg/kg body wt./day) and MCP I conjugated gold nanoparticle (200µg/kg body wt./day) containing nine male albino Wistar rats in each. Three animals were sacrificed monthly interval during 90 days of investigation period. No significant differences were examined in markers of blood serum i.e., cholesterol, creatinine, lactate dehydrogenase, creatine kinase, bilirubin, urea, triglyceride, HDL and concentrations of testosterone, luteinizing hormone and follicle stimulating hormone except drastic reduction in SGOT ($p < 0.001$) and SGPT ($p < 0.05$; $p < 0.01$; $p < 0.001$) in group C. The glycogen, cholesterol and lactate dehydrogenase level in testicular tissues, sialic acid and fructose corresponded to epididymal and seminal vesicles, respectively, demonstrated insignificant changes. Significant diminution was recorded in the level of L-carnitine and α -glucosidase at 60 days in group B ($p < 0.05$) and 30 days in group C ($p < 0.01$; $p < 0.001$) of epididymal tissues. Prostatic acid phosphatase was increased ($p < 0.05$; $p < 0.01$) in group C. While antioxidant biomarkers in testicular tissues viz., lipid peroxidase, superoxide dismutase, reduced glutathione and catalase were revealed insignificant alteration. Thus, status of biomarkers indicate that synergistic effect of MCP I conjugated gold nanoparticle was found to be targeted, safe and effective for oral contraception in male albino rats at highly reduced dosage compared to pure MCP I treated animals which may open new avenues for clinical development.

INTRODUCTION

Animal testing and experimentation intended to predict the safety and efficacy of new drugs from laboratory to clinics.^[1] It is very critical to address the cost and time for new therapeutics. Late stage trials usually increased cost of drug development resulted into 50% failure of phase III trial. Therefore, assessing of biomarkers has been multiplied in phase I studies.^[2] According to the National Institute of Health, biomarker is a characteristic that is objectively measured and evaluated

as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (e.g., DNA, protein, metabolite, mRNA or lipid). Recently, applications of biomarkers have been explored extensively for decision making at early stage of clinical development.^[3] Many investigators utilized the blood plasma of rat for profiling of metabonomic/metabolomic for the assessment of biomarkers as novel and sensitive tools for drug discovery.^[3,4] Moreover, researchers also assayed metabolome of rat plasma

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following 7, 14 and 28 days of treatment with statins, or fibrates and phenytoin (diphenylhydantoin).^[5,6] Regarded to this, it was also revealed that creatine kinase (CK) and lactate dehydrogenase (LDH) are associated with cardiac injury in animals which established troponin as a toxic marker for cardiac myocyte.^[7,8] It was demonstrated that renal function usually assessed through detecting the level of serum urea and creatinine but nowadays more sensitive biomarkers are being practiced *viz.*, urinary kidney injury molecules 1 (KIM-1), clustrin (CLU) and albumin closely associated with renal tubular alteration while urinary protein, cystatin C and β 2-microglobulin corresponded to glomerular damage of rat nephron.^[9] About fifteen fold elevated levels of alanine aminotransferase, aspartate aminotransferase or lactate dehydrogenase in blood serum indicate towards hepatopathy.^[10]

Nonetheless, wide utility and predictability of biomarkers, it has become less apparent in the field of reproductive medicines into translational research whereas numerous drugs have been approved. Accordingly, breakthrough using of biomarkers for drug development may pave the way to achieve goal of 3Rs. Recently, gold nanoparticles are being attracted towards drug delivery vehicles at the stage of preclinical/clinical, because high surface to volume ratio of particles make them better strategy for maximum adsorption of desired therapeutic molecules at targeted cellular organelles or tissues without altering immunogenicity.^[11] In this context, efficient delivery of hydrophobic nature of MCP I fraction derived from methanol sub-fraction of benzene chromatographic fraction of chloroform extract of *C. papaya* seed has been demonstrated through specific size of gold nanoparticle for improved male contraception in albino rats.^[12]

In this study, translational safety and efficacy of metabolomic profiles associated with blood serum (*e.g.*, cholesterol, creatinine, lactate dehydrogenase, creatine kinase, bilirubin, urea, triglyceride, HDL and concentrations of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH), SGOT and SGPT) and reproductive tissues (*e.g.*, the glycogen, cholesterol and lactate dehydrogenase level in testicular tissues, sialic acid and fructose corresponded to epididymal and seminal vesicles, respectively, L-carnitine and α -glucosidase, activity of acid phosphatase) While antioxidant biomarkers in testicular tissues *viz.*, lipid peroxidase, superoxide dismutase, reduced glutathione and catalase were assessed at the completion of 30, 60 and 90 days of treatment with orally administered pure MCP I and MCP I conjugated gold nanoparticle (AuNP-MCP I) in male albino rats.

MATERIALS AND METHODS

Gold Nanoparticles

The synthesis and physicochemical characterization of AuNP-MCP I nanoformulation and bioactive plant extract MCP I (methanol sub-fraction of benzene chromatographic

fraction of chloroform extract of *C. papaya* seed) has been reported. MCP I fraction was utilized as reducing, capping, stabilizing and contraceptive agents for synthesis of gold nanoparticle which was further examined for contraceptive efficacy in male Wistar albino rats.^[12]

Animal Treatment

Twenty seven male albino Wistar rats were equally divided in three experimental groups after acclimatization in plastic cages about two weeks. Proper hygienic conditions have also been maintained as per 12 hours light and dark cycles during investigation period of three months. Group A served as vehicle control; Group B orally treated with pure MCP I at the dose of 50 mg/kg body wt./day and Group C orally treated with AuNP-MCP I nanoformulation at the dose of 200 μ g/kg body wt./day for consecutive three months. Three animals were sacrificed from each group for the analysis of blood biomarkers and reproductive tissues according to the Institutional Animal Ethics Committee, University of Rajasthan.^[UDZ/IAEC/1/01/7/2017, 12, 13]

Biomarkers of Blood Serum

A portion of collected blood samples was used for serum separation which further used for biochemistry parameters: High density lipoprotein [HDL], triglycerides [TGL], bilirubin, urea, CK, LDH, serum glutamate oxalate transaminase [SGOT], serum glutamate pyruvate transaminase [SGPT], creatinine and cholesterol were estimated in semi auto analyser (ERBA-Smart Lab, Mumbai) using reagent kits (Transasia Biomedicals Ltd., Mumbai, India). The level of FSH, LH and testosterone in blood serum was examined by ELISA kits (Autobio Diagnostic Co. Ltd., Zhengzhou, China).

Biomarkers of Reproductive Tissues

Androgen sensitive biochemical markers were measured by UV-visible spectrophotometry according to the recommended protocol, *viz.*, glycogen,^[14] lactate dehydrogenase^[15] and cholesterol^[16] in testicular tissues, L-carnitine,^[17] α -glucosidase^[18] and sialic acid^[19] in epididymal tissues, the level of fructose and acid phosphatase in tissues of seminal vesicle and ventral prostate,^[20] respectively, were quantitatively estimated using tissue homogenates of all experimental animals.

Antioxidant Markers

Lipid peroxidase,^[21] catalase,^[22] superoxide dismutase,^[23] reduced glutathione^[24] were assayed in testicular tissues of all experimental animals.

Statistical Analysis

One-way analysis of variance test (ANOVA) with Holm-Sidak was used to calculate differences between mean values when compared to control group. Statistical analysis was carried out by software SPSS version 10.0 (SPSS Inc., Chicago, IL, USA) and variation among mean

values recorded to be significant when * $p < 0.05$; # $p < 0.01$; ● $p < 0.001$.

RESULTS

Gross Observation

The groups A, B and C animals did not show any sign of discomfort for a period of 90 days *viz.*, normal libido, mortality, morbidity, cage behaviour (skin, fur, eyes and nose), food and water intake, neurological symptoms (convulsions and tremors), autonomic activities (tiredness and salivation) bizarre behaviour such as walking backward, self mutilation, etc.), and diarrhea.

Biomarkers of Blood Serum

The level of cholesterol, creatinine, lactate dehydrogenase, creatine kinase, bilirubin, urea, triglyceride and HDL in blood serum of treated animals at 30, 60 and 90 days of investigation schedule exhibited insignificant changes compared to vehicle control.

However, serum concentration of SGOT drastically reduced ($p < 0.001$) in group C orally treated with AuNP-MCP I nanoformulation while the level of SGPT was revealed random diminution pattern ($p < 0.05$; $p < 0.01$ and $p < 0.001$) during the study period (Table 1).

Hormonal Profile

Monthly assessment of blood serum FSH, LH and testosterone hormone in animals treated with MCP I and AuNP-MCP I formulations after completion of 30, 60 and 90 days of experiment displayed no significant fluctuation throughout the investigation period when compared to vehicle control (Fig. 1P-R).

Biomarkers of Reproductive Tissues

The glycogen, cholesterol and lactate dehydrogenase level in testicular tissues, sialic acid contents in epididymal tissues and fructose contents in seminal vesicles following 30, 60 and 90 days of treatment have been demonstrated insignificant changes compared to vehicle control during investigation of three months. Whereas other markers corresponded to the level of L-carnitine and α -glucosidase following 60 days of experiment showed significant diminution ($p < 0.05$, $p < 0.001$) in group B while group C displayed this pattern after 30 days ($p < 0.05$, $p < 0.01$, $p < 0.001$) of treatment. Activities of acid phosphatase were increased ($p < 0.01$, $p < 0.05$) in group C following 60 of treatment (Fig. 2E-L).

Antioxidant Markers

Oxidative damage to the testis was assessed using enzymatic (*viz.*, catalase, superoxide dismutase and lipid peroxidase) and non-enzymatic (reduced glutathione) tissue biomarkers. The level of lipid peroxidase, superoxide dismutase and reduced glutathione in testicular tissues were examined after completion of experiment during

Table 1: Biomarkers of blood serum of albino male rats following orally treated with free MCP I and MCP I conjugated gold nanoparticle for a period of 90 days.

Serum markers	30 days			60 days			90 days		
	Experimental groups								
	A	B	C	A	B	C	A	B	C
Cholesterol (mg/dl)	114.93 ± 5.88	112.03 ± 4.97	127.90 ± 8.13	116.80 ± 11.11	113.70 ± 5.75	126.46 ± 12.55	120.03 ± 7.31	114.26 ± 5.01	128.76 ± 5.88
Creatinine (mg/dl)	0.59 ± 0.044	0.68 ± 0.072	0.61 ± 0.043	0.67 ± 0.012	0.56 ± 0.004	0.61 ± 0.073	0.56 ± 0.081	0.64 ± 0.018	0.60 ± 0.108
SGPT (IU/L)	32.56 ± 3.13	30.88 ± 3.19	17.46 ± 1.59●	30.73 ± 1.60	34.66 ± 3.83	20.40 ± 1.18*	34.20 ± 1.21	31.30 ± 3.81	18.66 ± 2.52 [#]
SGOT (IU/L)	38.80 ± 2.46	39.40 ± 0.88	11.43 ± 1.18●	36.53 ± 2.30	38.23 ± 3.06	12.83 ± 1.24●	39.86 ± 2.20	38.60 ± 2.19	13.30 ± 0.97●
Lactate dehydrogenase (IU/L)	318.66 ± 4.92	328.00 ± 6.16	316.00 ± 3.26	324.00 ± 1.63	323.66 ± 3.85	320.33 ± 2.49	317.33 ± 4.64	319.66 ± 2.49	316.33 ± 4.49
Creatine kinase (IU/L)	205.33 ± 11.02	193.66 ± 12.11	191.00 ± 11.77	199.33 ± 10.62	209.00 ± 8.04	215.33 ± 12.71	207.33 ± 9.17	196.00 ± 10.67	204.33 ± 8.17
Bilirubin (mg/dL)	0.78 ± 0.079	0.87 ± 0.061	0.85 ± 0.045	0.73 ± 0.037	0.80 ± 0.049	0.87 ± 0.151	0.78 ± 0.037	0.87 ± 0.098	0.70 ± 0.029
Urea (mg/dL)	27.10 ± 1.93	29.23 ± 5.16	25.80 ± 1.47	26.06 ± 1.11	30.00 ± 1.57	25.40 ± 0.92	28.26 ± 2.87	27.43 ± 1.21	28.50 ± 0.88
Triglyceride (mg/dL)	88.00 ± 1.39	87.50 ± 1.18	82.33 ± 9.94	95.70 ± 2.47	78.26 ± 6.57	94.60 ± 2.80	86.93 ± 1.18	86.73 ± 3.32	91.46 ± 7.64
HDL (mg/dL)	41.80 ± 0.35	40.40 ± 2.40	42.53 ± 0.75	39.43 ± 2.29	41.16 ± 3.51	42.83 ± 0.98	40.73 ± 1.22	42.93 ± 0.46	42.23 ± 0.24



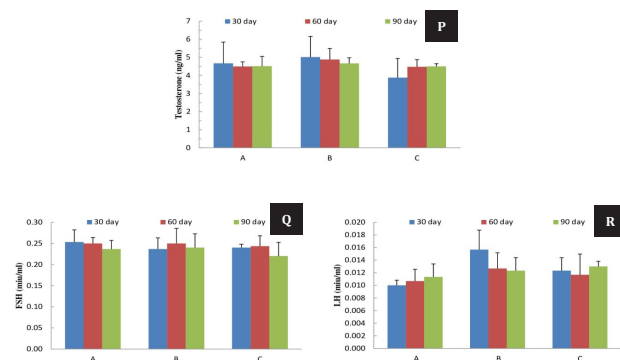


Fig. 1P-R: Hormonal profile of albino male rats treated orally with pure MCP I and AuNP-MCP I for a period of 90 days. Group A: vehicle control; Group B: MCP I fraction @ 50 mg/kg body wt/day; Group C: MCP I conjugated gold nanoparticles @ 200 µg/kg body wt/day

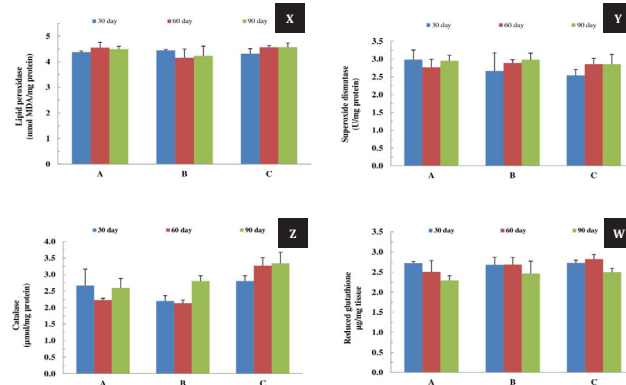


Fig. 3X-W: Analysis of antioxidant biomarkers of testicular tissues of albino rats treated orally with pure MCP I and AuNP-MCP I for a period of 90 days. Group A: vehicle control; Group B: MCP I fraction @ 50 mg/kg body wt/day; Group C: MCP I conjugated gold nanoparticles @ 200 µg/kg body wt/day

DISCUSSION

Obtained results of this study, preclinical safety and efficacy of AuNP-MCP I nanoformulation was investigated using specific candidate biomarkers corresponding to major vital organs viz., kidney, liver, heart and accessory gland (seminal vesicle and prostate). Experimental findings greatly support the biocompatible nature of this nanoformulation regarding to unaltered biomarkers compared to vehicle control animals. Recently, Parveen *et al.*^[25] observed similar findings on the level of biomarkers including creatinine, total cholesterol, blood urea, creatinine, creatine kinase, triglycerides, alanine amino transferase, aspartate amino transferase, alkaline phosphatase in rats treated with functionalized gold nanoparticles with aqueous extract (*S. aromaticum*) during a period of 28 days. In this study, the level of L-carnitine and α -glucosidase declined significantly following 60 days ($p < 0.05$, $p < 0.001$) in group B whereas group C displayed this pattern after 30 days ($p < 0.01$, $p < 0.001$) of treatment. Findings suggest that declined level of L-carnitine was found to be corresponding to the loss of sperm motility in AuNP-MCP I treated group.^[26,27,14] Furthermore, significant diminution in the activity of α -glucosidase indicates deterioration in the process of sperm maturation of treated animals.^[28] Activity of acid phosphatase in prostatic tissues was found to be higher in group C treated with nanodrug of AuNP-MCP I when compared to group B animals treated with MCP I fraction, and respective vehicle control. Functional activity of rat prostate did not affect when intraperitoneal injection of citrate stabilized gold nanoparticle conjugated ascorbic acid (size ranges 10–15 nm; spherical) using 1 mg/kg dose for 10 days whilst same size of sodium polyphosphate stabilized silver nanoparticles deteriorate functional status of prostate.^[29] It was indicated that surface coating with MCP I fraction on gold nanoparticles may be played an important role for the novel contraceptive properties.

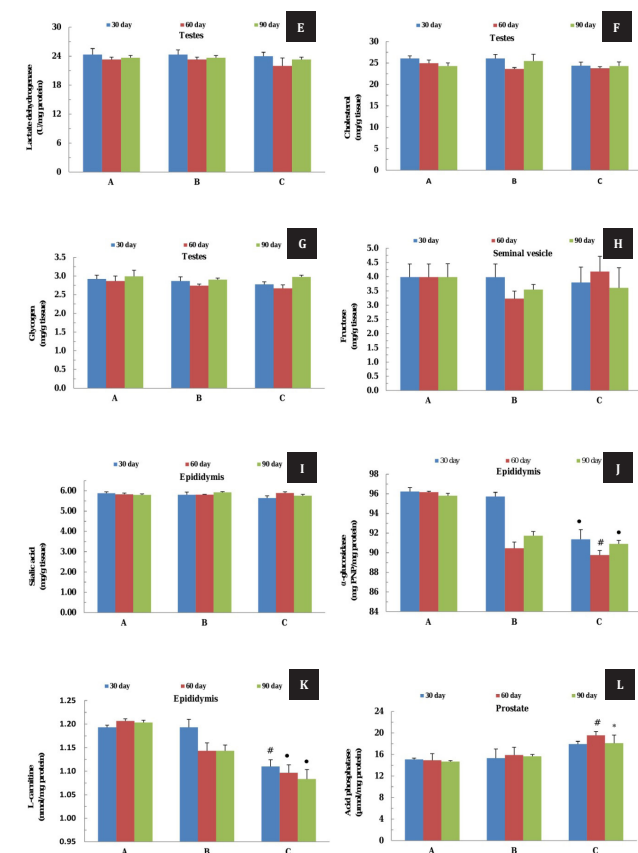


Fig. 2E-L: Analysis of tissue biomarkers of male reproductive system of albino rats treated orally with pure MCP I and AuNP-MCP I for a period of 90 days. Group A: vehicle control; Group B: MCP I fraction @ 50 mg/kg body wt/day; Group C: MCP I conjugated gold nanoparticles @ 200 µg/kg body wt/day

30, 60 and 90 days which revealed insignificant alteration compared to respective control. While catalase activities in testicular tissues were enhanced insignificantly in group C during 90 days of investigation period (Fig. 3X-W).

Additionally, positive effects on antioxidant markers have been demonstrated in animals when administered with AuNP-MCP I nanoformulation. In accordance with this, no deleterious effects have been displayed in level of antioxidant markers of testicular tissues consisting of enzymatic (*viz.*, catalase, superoxide dismutase and lipid peroxidase) along with non-enzymatic (reduced glutathione). The activity of catalase enzyme in group C was found to be at insignificantly increased level at 90 days of investigation. Similar ameliorative properties against antioxidant markers of testicular tissues observed after oral treatment of zinc oxide nanoparticles at a dose of 10 mg/kg/day in diabetic rats during 30 days of investigation period.^[30] Enhanced antioxidant biomarkers such as reduced glutathione, superoxide dismutase and catalase ($p < 0.05$) were recorded when orally ingested anaemic mice treated with a dose of 0.25 mg/kg fenugreek seed extract conjugated gold nanoparticle (spherical particle size ranges 5–20 nm).^[31] Furthermore, selenium nanoparticles displayed positive impact on male reproductive system of rats treated with dose of 0.2, 0.4, or 0.8 mg Se/kg body weight for a period of two weeks without altering the level of antioxidant enzymes.^[32] It has been clarified that plant extract inspired nanoparticles did not disrupt antioxidant status of testicular tissues. Accordingly, epididymal tissue biomarkers, *i.e.*, α glucosidase and L-carnitine were significantly declined in treated animals that coincides with increased sperm anomalies, diminution in sperm count, motility and viability after treatment with AuNP-MCP I nanoformulation.^[12] These reports indicate that AuNP-MCP I nanoformulation also support positive correlation between epididymal tissues markers and impaired sperm parameters for the effective contraception in albino rats.

Hepatotoxicity usually assessed by evaluating the level of alanine aminotransferases (ALS) and aspartate aminotransferases (AST) in blood serum. There was no alteration observed in biomarkers of blood serum following oral administration of AuNP-MCP I nanoformulation in all treated animals except significant reduction was observed in SGOT or AST and SGPT or ALT. Accordingly, oral gavage to silver/gold nanoparticles at treatment doses of 10, 50 and 100 mg/kg for 30 days decreased the level of AST and ALT in blood serum of rats in comparison to control animals.^[33] Obtained results may be decreased due to inhibition of enzyme activities following interaction of nanoparticles because Au or Ag nanoparticles have been demonstrated their potential towards modulation of protein structure reacting with thiol functional group.^[34,35] Hormonal level of testosterone, FSH and LH in blood serum of treated animals with AuNP-MCP I nanoformulation found to be insignificant change compared control group. Regarded to this, Behnammorshedi *et al.*^[36] demonstrated that gold nanoparticles functionalized with citrate and orally

administered to rats at doses of 25, 50, and 100 ppm for a period of 10 days significantly increased level of LH, FSH and testosterone. While gold nanoparticle functionalized with PEG-NH₂ using a single injection via the tail vein at the doses of 45 mg/kg (particle size 14 nm) enhanced serum testosterone levels without affecting fertility of mice whilst no alteration revealed in the level of FSH and LH.

POSSIBLE MODE OF ACTION

In this experiment, nanoformulation consisting of AuNP-MCP I possesses novel contraceptive potential due to its increased absorption at targeted testicular tissues with sustained release character and altered in tissue distribution. As it disrupts the process for sperm formation known as spermatogenesis. It was illustrated that the sperm maturation process was specifically intervened by reducing the level of tissue markers *i.e.*, α -glucosidase and L-carnitine in epididymis. A little fluctuation in these markers may lead to the production abnormal sperms resulted into loss of motility, viability and density of sperms^[12]. Contraceptive efficacy of this nanoformulation is directly corroborated with the presence of sufficient number of functional spermatozoa in cauda epididymal fluid. However, further in vitro experimentation is needed to verify the fertilizing ability of sperms in order to define precise mechanism for this nanoformulation using more educated biomarkers.

CONCLUSION

Safety and efficacy of AuNP-MCP I nanoformulation have also been examined on the basis of specific tissues/blood markers which clearly validate our findings towards male contraception. No adverse effects were illustrated in hormonal profile, level of antioxidant in testicular tissues and biochemical parameters of blood serum during investigation period. Advantages of this method may open the new avenues for translational research of other herbal products as well as synthetic agents with low therapeutic index. However, this is preliminary investigation employing markers of blood and reproductive tissues for evaluation of contraceptive drug consisting of AuNP-MCP I nanoformulation. Hence, additional experimentation is also needed on different animal species for further approval this potential male contraceptive approach using more educated, convenient and cost effective biomarkers utilizing by 'omics technologies' to attain the goal of 3R's.

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