Biomedicine & Pharmacotherapy 133 (2021) 111085

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

The effect of *Nigella sativa* oil and metformin on male seminal parameters and testosterone in Wistar rats exposed to an obesogenic diet

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ARTICLE INFO

Keywords:
Metformin
Nigella sativa
Obesity
Semen analysis
Testosterone

ABSTRACT

Obesity is a significant global health and socio-economic challenge, and considered an important risk factor for poor health outcomes including male reproductive dysfunction and infertility. As excess adiposity causes testicular dysfunction and infertility, novel therapeutic strategies require investigation. *Nigella sativa* (Ns) seed oil and metformin have both demonstrated a potential positive effect on obesity, although both remain poorly investigated in male fertility. Therefore, this study aimed to determine the effect of Ns oil and metformin on total body weight (TBW), mitochondrial membrane potential (MMP), serum testosterone and semen parameters in an obese animal model. Wistar rats (n = 54) were divided into six groups: normal chow (NC), high sugar diet (HSD) only, HSD and saline, HSD and metformin (75 mg/Kg/day), HSD and Ns (200 mg/Kg/day) (NS200), HSD and Ns (400 mg/Kg/day) (NS400). Intervention was force fed for the last 8 weeks of the 14 weeks dietary exposures. Results showed that the HSD increased TBW ($P = 0.001$) and reduced sperm concentration ($P = 0.013$) and progressive motility ($P = 0.009$) compared to the NC group. Metformin, NS200 and NS400 improved TBW ($P = 0.035$, $P = 0.006$ and $P = 0.005$, respectively) and testosterone ($P < 0.001$) compared to the HSD saline group, where metformin and NS400 improved sperm concentration ($P < 0.001$ and $P = 0.049$, respectively) and MMP ($P < 0.001$). There were no changes in sperm motility and viability for all experimental exposures, although NS400 ($P = 0.047$) negatively affected sperm viability. Metformin and Ns may be novel treatment options in obesity-induced infertility, although a potential negative impact on viability is cautioned for high dose Ns. These results warrant further investigation of Ns and Metformin for the management of obese infertile males.

1. Introduction

Obesity is clinically defined as a body mass index (BMI) > 30 Kg/m^2, and is considered a significant global health challenge that is driven by genetic, environmental, social, economic and cultural influences [1]. As a health challenge, there are numerous well defined complications of obesity, including metabolic syndrome, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and various malignancies [2]. Evidence strongly suggests that obesity has a negative impact on male reproduction and fertility potential, mediated through diverse mechanisms common to the complex metabolic, immune and endocrine dysfunctions associated with obesity [3,4]. This important relationship between obesity and male infertility has led to a demonstratable increase in scientific interest on the topic over recent years [5].

Obesity negatively impacts sperm concentration, motility, viability, normal morphology and DNA integrity [2,4]. Endocrine changes include male hypogonadism, mediated through hypothalamic and testicular dysfunction, alongside hyperinsulinaemia, hyperleptinaemia and hyperestrogenaemia [3,4]. Systemic low grade inflammation and oxidative stress are further important derangements that may contribute to reproductive dysfunction [2]. In addition, paternal obesity increases the risk for metabolic and neurological pathology in the offspring, mediated through altered epigenetic regulation of gene expression in the male germ cells acquired during spermatogenesis [3]. With limited management options, novel therapeutic strategies that favourably modulate the complex underlying mechanisms common to obesity and male infertility require investigation, including the use of metformin and medicinal plants (phytotherapy) [2]. Various medicinal plants have

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https://doi.org/10.1016/j.biopha.2020.111085
Received 3 August 2020; Received in revised form 6 November 2020; Accepted 28 November 2020
Available online 8 December 2020

been reported in a recent systematic review to have evidence for a beneficial effect in obesity, including *Camellia sinenesis*, *Caralluma fimbriata*, *Garcinia cambogia*, *Irvingia gabonensis, Nigella sativa* and *Phaseolus vulgaris* [6].

Nigella sativa (Ns), commonly known as black cumin or black seed in English and *Habbat al-barakah* in Arabic, is a member of the Ranuculaceae family. It is an annual herbaceous plant with characteristic white, green, yellow or pink flowers of 5–10 petals, with black trigeminal seeds produced within the fruits. The plant is native to Southwest Asia, the Middle East and Mediterranean countries of Africa and Europe [7], where Ns seeds have been used extensively in human history for culinary and medicinal purposes dating back over 1400 years [8]. The seeds are described as aromatic and bitter to taste, and composed of oils (±40%), proteins (±21%), carbohydrates and fibres (±8.4%), ash (±4.5%) and moisture (±3.8%) [8]. Traditional uses of Ns, recorded particularly in Unani traditional medicine and Islamic medicine, include obesity and complications such as hypertension and diabetes. Scientific investigation in both *in vitro* and *in vivo* studies have demonstrated anti-inflammatory, anti-oxidant, anti-microbial and anti-carcinogenic (anti-proliferative and pro-apoptotic) effects, and improvement in atherogenesis, endothelial dysfunction, dyslipidaemia, insulin regulation and glucose intolerance [7–9]. Human clinical studies have shown potential therapeutic effects in obesity, metabolic syndrome, cardiovascular disease, T2DM and reproductive disorders. Importantly, a recent meta-analyses found good evidence for the use of Ns in human obesity and relevant co-morbidities, although there is reportedly a high heterogeneity of the limited studies included for analysis [10–12].

Metformin (*N,N*-dimethylbguanide) is a biguanide derivative that primarily improves insulin sensitivity in diabetic and pre-diabetic (insulin resistant) patients, particularly in patients with clinical obesity [13]. Metformin also reduces the incidence of metabolic syndrome in overweight patients [14]. This is primarily achieved through the reduction of intestinal glucose absorption and hepatic gluconeogenesis mediated by improved insulin regulation [13]. Studies have suggested a potential benefit of metformin for male reproductive dysfunction associated with obesity and insulin resistance, although this requires significant further investigation [15].

As metformin and Ns are suggested to improve obesity, and may have potential benefit in male reproductive parameters, this study aimed to investigate the effect of metformin and Ns oil on the male reproductive system in an animal model of obesity.

2. Materials and methods

2.1. Study overview and design

Male Wister rats (n = 48) weighing 180–220 g were obtained from Tygerberg Hospital (Tygerberg, South Africa) and housed in standard cages within the animal facilities unit of the Department of Medical Bioscience (MBS), University of Western Cape (UWC) (Bellville, South Africa). The animals were housed under controlled temperature between 21–23 °C, and an artificial 12-h light/dark cycle. Principles of animal care were conducted in accordance with the South African National Standard (SANS 10386:2008) for The Care and Use of Animals for Scientific Purposes. Ethical clearance was obtained from the Senate Research Committee (UWC) (Ethics Clearance Registration no: 15/4/102). An overview of the groups and study design is provided in Fig. 1.

The rats were randomly divided into six groups (n = 8 per group). Five groups received an obesogenic high sugar diet (HSD) [16,17], and one group received normal rat chow (lean group), for 14 weeks. The lean group was compared to one HSD group without any intervention or control administered. The remaining four HSD groups were used for the experimental exposures (*Nigella sativa* high and low dose groups, and a metformin group) and the saline control group. The HSD consisted of rat chow (Petco, Bellville, South Africa) soaked in water, into which sucrose was dissolved. This was then mixed with condensed milk (Ndlovu Corp

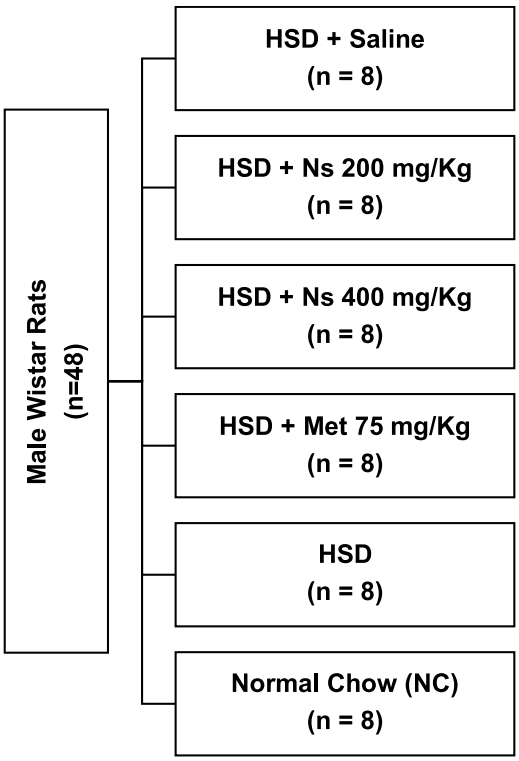

Fig. 1. Overview of the experimental design of the study. Following 14 weeks of HSD including 8 weeks' intervention for the experimental groups, total body weight, semen parameters and serum testosterone was investigated. HSD = high sugar diet; Ns = *Nigella sativa* oil.

Supply, Montague Gardens, South Africa) to produce soft food. The final ratio was 33% rat chow, 33% condensed milk, 7% sucrose and 27% water. Food and water were freely available to the rats throughout the study.

No intervention was provided for the first 8 weeks during the 14 weeks of HSD exposure. This was followed by 6 weeks of experimental exposures in the four groups, namely: control (saline), *Nigella sativa* 200 mg/Kg/day (NS200), *Nigella sativa* 400 mg/Kg/day (NS400) and metformin 75 mg/Kg/day. Ns oil was purchased from Crede Natural Oils (Cape Town, South Africa) as a cold pressed extraction from the seeds. Metformin was obtained through Adcock Ingram (Johannesburg, South Africa). The dosages of Ns and metformin were based on previous reports [18–21]. Calculations of individual dosages were done daily based on the animal weights, and they were force fed respective exposures and control *via* oral gavage. The control group was given 100 µL of 0.9 % saline solution only, prepared by dissolving 9 g of NaCl (Sigma-Aldrich, Steinheim, Germany) to 1 l of deionised water. The animals were further monitored, weighed and physically inspected for signs of distress daily.

Following the completion of the experimental phase, the animals were euthanized by rapid cervical dislocation as described by Solomon and colleagues [22], and tissues obtained for data analysis. Variables for statistical analysis included total body weight, spermatozoa parameters (concentration, motility, viability and mitochondrial membrane potential) and serum testosterone.

2.2. Determination of semen parameters

The head of the caput epididymis was isolated and placed in a petri dish (35 × 10 mm) containing 1 mL Dulbecco's Modified Eagle's Medium DMEM (Gibco, Auckland, New Zealand), and supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, USA). A total of 1 mL of the medium was placed in a second petri dish, and the distal caudal epididymis was isolated and incubated at 37 °C (Gwayi & Bernard,

2002). A slit was made in the cauda to allow the sperm to swim out into the medium for about 10 s in order to collect spermatozoa for analysis.

Sperm concentration and motility was determined using the Computer Assisted Semen Analysis (CASA) equipment and software (SCA® version 6.0) (Microptic S.L, Barcelona, Spain). A Basler A312FC digital camera, mounted onto a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), was used for all CASA analyses. The microscope was provided with a 10X objective, phase contrast optics and a pre-heated stage of 37 °C. In order to analyse the semen sample, 2 μl of the spermatozoa obtained from the swim out from the cauda epididymis was prepared in medium at 37 °C and then placed onto a Leja slide (Leja, Nieuw Vernep, The Netherlands). Thereafter, the slide was viewed under a microscope and a minimum of 200 motile spermatozoa over a minimum of two different frames from every 4th microscopic field were analysed for each sample. The average of two different analyses was considered for statistical analysis.

Sperm vitality was assessed using the eosin-nigrosin stain. For each sample, 50 μl of eosin-nigrosin stain was mixed gently with 50 μl of a spermatozoa sample separately, and 20 μl of the mixture was then smeared to a microscopy slide. After drying, the slide was analysed under the microscope (Zeiss, Oberkochen, Germany) at 100x magnification. A total of 200 spermatozoa were counted on each slide, and the percentage of live (viable) sperm was calculated from spermatozoa that remained white (indicates an intact cell membrane with the stain). Dead spermatozoa appeared pink or red (stain taken up into the cell due to a damaged cell membrane)

Sperm mitochondrial membrane potential (MMP) was assayed using the DePsipher staining kit (Trevigen, Gaithersburg, USA). In brief, spermatozoa were re-suspended in 50 μl of DePsipher solution and incubated for 20 min at 37 °C in the dark. After incubation, 10 μl of each sample was viewed with 488 nm excitation and 590 nm emission filters at 1000 x magnification with oil immersion on an epifluorescence microscope (Zeiss, Oberkochen, Germany). Cells with disturbed MMP showed green fluorescence with an emission of 520 nm, whilst spermatozoa with intact MMP showed red fluorescence at 590 nm. An average of 200 spermatozoa was manually counted to determine a percentage (red cells divided by total cells counted) of spermatozoa with intact MMP (red) for data analysis.

2.3. Determination of serum testosterone

Following cervical dislocation, a blood sample was obtained from cardiac puncture, and allowed to clot for 30 min in 1.5 mL micro-centrifuge tubes (Corning, New York, USA). This was then centrifuged at 3000 × g for 15 min and serum was separated and stored at −20 °C until biochemical evaluation. Testosterone concentration was determined using the Testosterone ELISA kit (DRG Instruments GmBH, Marburg, Germany). The assay was performed according to the manufacturer's instructions. In brief, 25 μl of each standard and sample was added into each well containing 200 μl enzyme conjugate and incubated for 60 min at room temperature without covering the plate. Following incubation, the wells were washed 3 times with washing buffer. Residual droplets were removed, and then 200 μl of substrate solution was added to each well and incubated for a further 15 min at room temperature. The reaction was stopped by adding 100 μl stop solution to each well and the signal was read at 450 nm using a Microplate Reader Model LT-4000 (Labtech, East Sussex, UK). Using the standard curve, the concentration of testosterone in the experimental samples was calculated for data analysis. The obtained results are expressed as ng/mL.

2.4. Statistics

Statistical analysis was carried out by using MedCalc statistical software (Version 12.1.3.0, Mariakerke, Belgium). Normal distribution was checked by Kolmogorov-Smirnoff test, and the variables compared by an independent sample *t*-test or the Mann-Whitney test depending on the normal or abnormal data distribution, respectively. Data is represented as bar graphs with standard deviation for parametric data or box-and-whisper graphs for non-parametric data. A P-value of < 0.05 was considered statistically significant

3. Results

3.1. Total body weight

The HSD group total body weight was significantly heavier compared to the lean group ($P = 0.0001$). Metformin ($P = 0.0345$), NS200 ($P = 0.0057$) and NS400 ($P = 0.0048$) significantly improved total body weight compared to saline. There was no significant difference between metformin and NS200 or NS400 (Fig. 2).

3.2. Semen parameters

The sperm concentration was significantly lower in the HSD group compared to the lean group ($P = 0.0128$). Metformin significantly improved sperm concentration compared to saline ($P = 0.0003$). NS200 caused a non-significant increase ($P = 0.1043$), whereas the NS400 group showed a significant increase for sperm concentration compared to saline ($P = 0.0493$). Metformin further showed a significantly higher sperm concentration as compared to the NS200 ($P = 0.0168$) and NS400 ($P = 0.0295$) groups (Fig. 3).

Progressive motility was significantly lower in the HSD group compared to the lean group ($P = 0.0092$), with no significant difference for non-progressive motility ($P = 0.9655$). There was no significant difference in progressive motility for metformin ($P = 0.3865$), NS200 ($P = 0.2332$) and NS400 ($P = 0.8253$) groups compared to saline. Similarly, there was no significant difference in non-progressive motility between for metformin ($P = 0.5414$), NS200 ($P = 0.1198$) and NS400 ($P = 0.5895$) groups compared to saline. Non-progressive motility was significantly lower in the NS200 group compared to metformin ($P = 0.0355$), but not the NS400 group ($P = 0.2702$) (Fig. 4 and 5).

Sperm viability did not vary significantly in the HSD group compared to the lean group ($P = 0.9648$). There was no statistical difference in sperm viability between the metformin and saline group ($P = 0.5966$), while NS200 showed a non-significant decrease in sperm viability ($P = 0.0637$), with a significant decrease for NS400 ($P = 0.0469$). Moreover, no significant difference between the metformin and the NS200 ($P = 0.1489$) or NS400 ($P = 0.1234$) groups was observed (Fig. 6).

The MMP was not significantly different in the HSD group compared to lean group ($P = 0.2808$). Metformin significantly improved MMP compared to saline ($P = 0.0003$), while NS200 showed a non-significant increase in MMP ($P = 0.3067$). The administration of 400 mg Ns/Kg/day (NS400) resulted in a significant increase in the percentage of MMP-intact sperm ($P < 0.0001$). In the metformin group, the percentage of MMP-intact sperm was significantly higher compared to the NS200 ($P = 0.0003$) group, with no statistical difference with NS400 ($P = 0.8942$) (Fig. 7).

3.3. Serum testosterone

The serum testosterone concentration was non-significantly higher in the HSD group compared to the lean group ($P = 0.4599$). In contrast, metformin ($P = 0.0001$), NS200 ($P = 0.0002$) and NS400 ($P < 0.0001$) administration led to significantly increased serum testosterone concentrations compared to the saline group. There was no significant difference between the metformin group and the NS200 ($P = 0.3649$) and NS400 ($P = 0.7948$) groups (Fig. 8).

4. Discussion

Obesity has been established to have a negative impact on male reproduction [2–4]. In humans and animals, high energy sugar diets

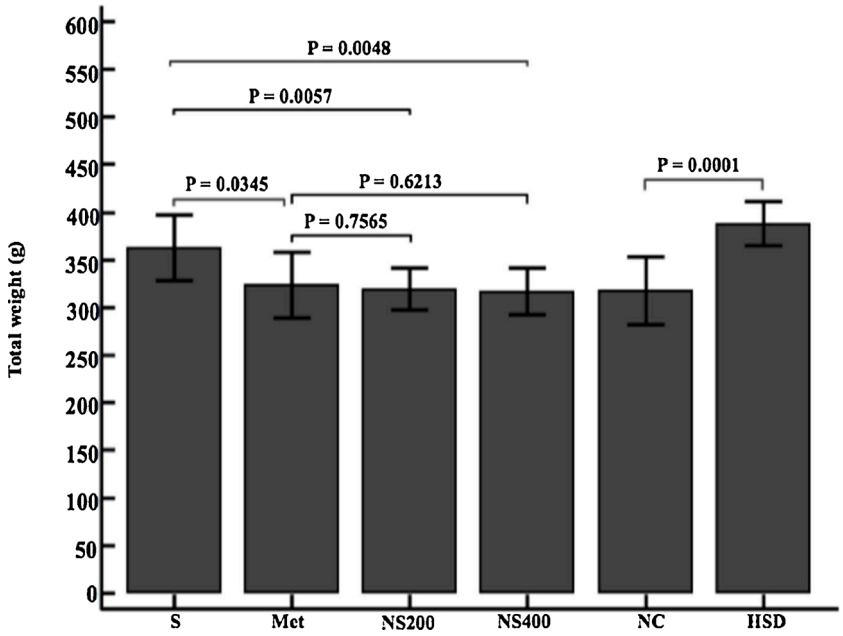

Fig. 2. Comparative analysis of the groups for total body weight. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

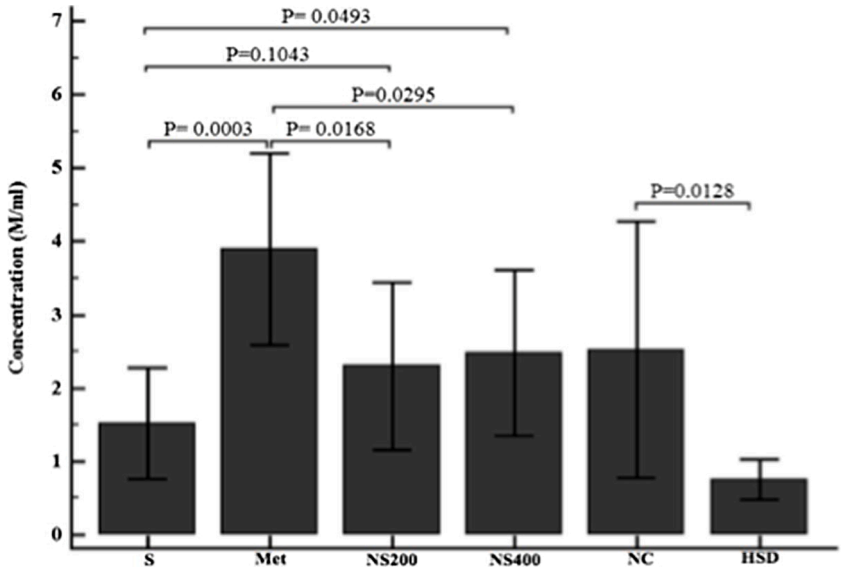

Fig. 3. Comparative analysis of the groups for sperm concentration (M/mL). S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

induce obesity and associated metabolic co-morbidities (dyslipidaemia, hyperglycaemia and hyperinsulinaemia) and complications (CVD and T2DM) [16,23–27]. As expected, the results of this study showed that the HSD diet resulted in a significant increase in total body weight compared to the lean control consuming normal chow. The HSD group further showed a significant decrease in sperm concentration and progressive motility, with no significant effect on non-progressive motility, viability or MMP. These results support the findings of Adekunbi et al. (2016), showing a decline in sperm concentration, motility, viability, morphology and serum testosterone using a high sucrose diet in rats [28]. Interestingly, the HSD group did not show reduced serum testosterone concentrations compared to the lean group, where rats fed high sucrose or fructose diets have been previously reported to have reduced serum testosterone concentrations [28,29]. In this context, Ns seed oil and metformin may improve obesity and the negative impact of obesity on male reproduction [7–12,14,15]. This study further investigated metformin and Ns oil as a potential management option for obesity induced male reproductive dysfunction in this HSD animal model.

Metformin is an anti-hyperglycaemic medication commonly prescribed in T2DM and insulin resistance associated with obesity and metabolic syndrome [13,14]. Although metformin has been widely studied and reported to result in weight loss in non-diabetic patients, its effects on the male reproductive system are not well known [15,30]. However, in obese and insulin resistant men, metformin appears to improve reproductive parameters of males with T2DM [15]. In this study, a significant decrease in total body weight was reported in the metformin group, consistent with the benefit of metformin in obese diabetic and pre-diabetic patients [13,14]. Metformin treatment further showed significant improvements in sperm concentration and MMP-intact spermatozoa, with no significant effect on motility or viability, alongside

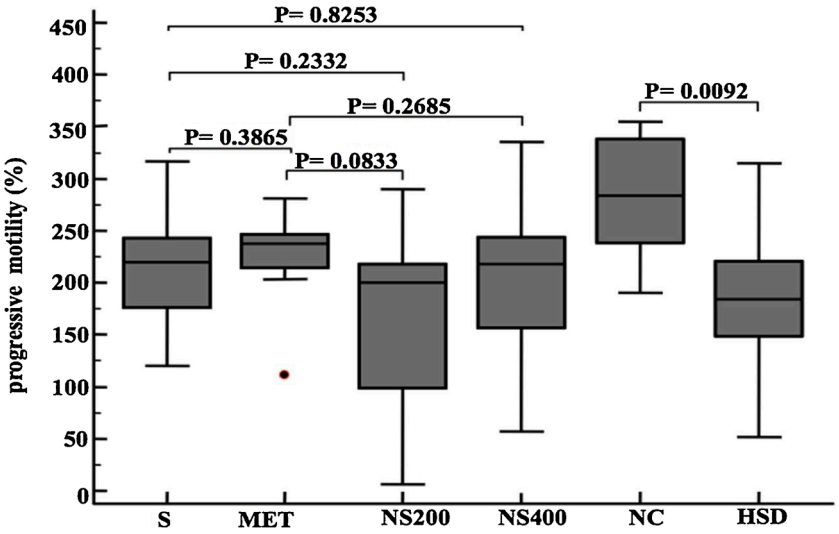

Fig. 4. Comparative analysis of the groups for progressive motility (%). S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal chow group; HSD = High Sugar Diet group.

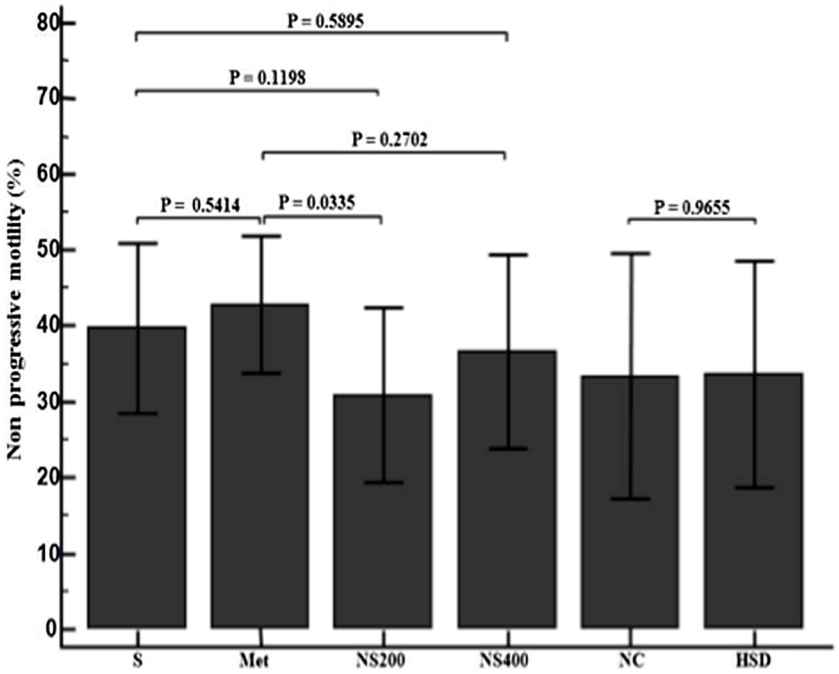

Fig. 5. Comparative analysis of the groups for non-progressive motility. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

improved serum testosterone levels. These results support limited studies where metformin significantly improves sperm parameters, increases testosterone and reduces estrogen concentrations in obesity [14,31]. Furthermore, metformin has been shown to improve epididymal sperm quality and antioxidant function in the testes, including increased superoxide dismutase (SOD) and glutathione peroxidase [32]. In humans, metformin treatment for 6 months has been suggested to improve oligoasthenoteratozoospermia in patients with metabolic syndrome [33]. Further evidence in streptozotocin (STZ) induced diabetic animal models supports the use of metformin in improving sperm parameters, sperm DNA integrity and epididymal sperm count, reduces lipid peroxidation and increased endogenous antioxidant concentrations, and increases intra-testicular testosterone levels alongside serum testosterone, LH and FSH concentrations [34–37]. However, in healthy animal models, metformin appears to negatively affect reproductive functions, including epididymal sperm concentration and motility, causing increased testicular lipid peroxidation and reduced SOD, catalase and glutathione activity [38]. In an *in vivo* experiment in which ejaculated human sperm was exposed to metformin, there was a reduction in total motile and progressively motile spermatozoa, with no effect on viability, MMP or mitochondrial superoxide generation. This was mediated through inhibition of the protein kinase pathway and protein tyrosine phosphorylation [39]. Similarly to metformin, myo-inositol's are potential insulin sensitizing and antioxidant formulations for male infertility management, which have been suggested for clinical use particularly in assisted reproduction techniques [40,41]. This further suggest that modulation of insulin sensitivity may be an important target for obesity induced male infertility.

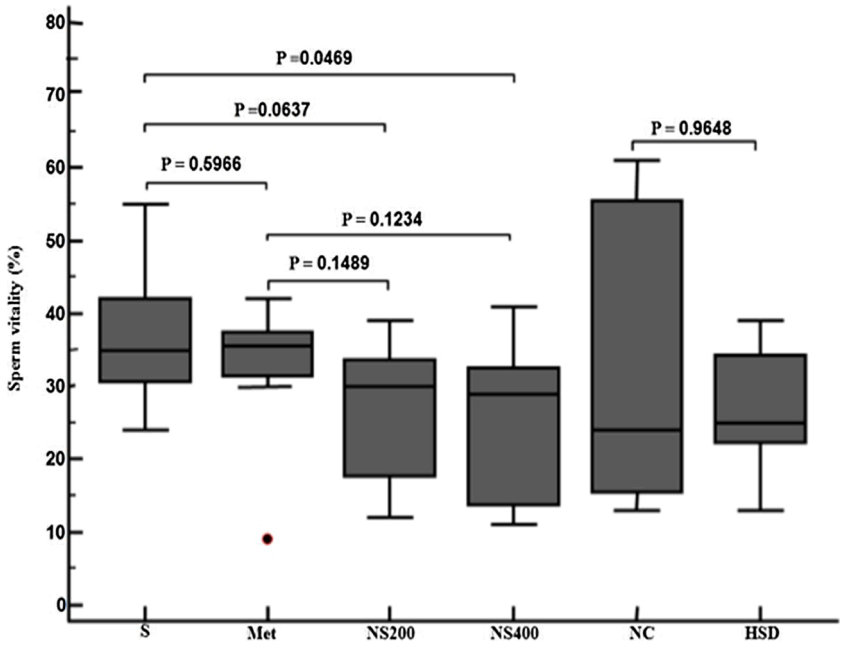

Fig. 6. Comparative analysis of the groups for sperm viability (% viable). S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

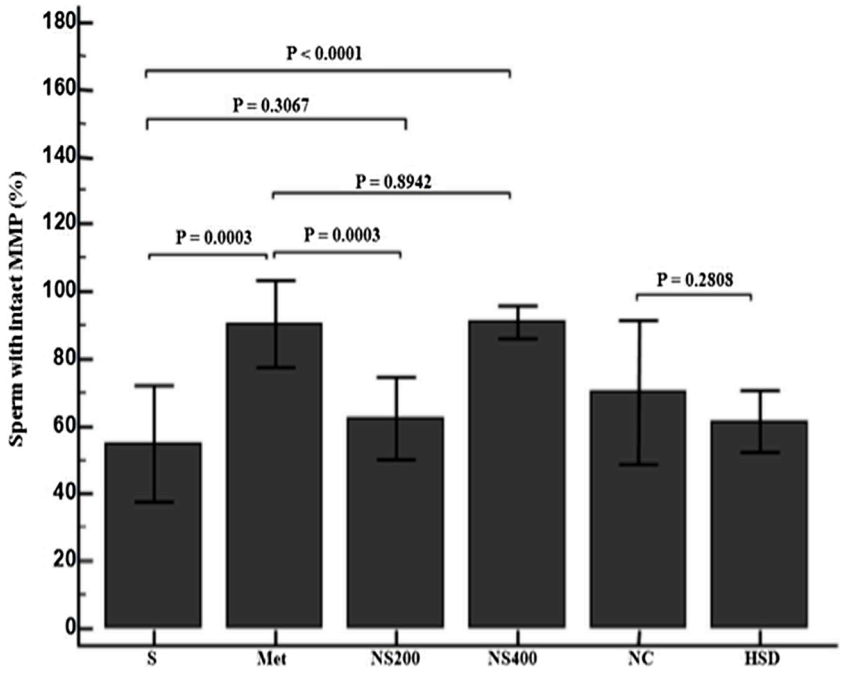

Fig. 7. Comparative analysis of the groups for MMP (% intact). S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Ns is a herbal medicine that has a wide use of applications in traditional medicine systems. Traditional and scientific evidence suggests that Ns seed oil may be beneficial in obesity and associated complications, with the notion that Ns may also improve male reproductive parameters [7–12]. Similarly to metformin, the results of this study showed that Ns oil improved body weight, sperm concentration, percentage of MMP-intact sperm, and serum testosterone levels with no significant effect on progressive and non-progressive motility. However, there remains a paucity of studies investigating the potential use of Ns oil on obesity induced impaired reproductive parameters. In a HFD induced model of hyperlipidaemia in rats, Ns oil extract (0.5 mL/Kg) improved sperm concentration, motility, morphology and serum testosterone concentrations [42]. In an isolated randomised controlled trial of Iranian infertile men with abnormal semen parameters, Ns oil treatment (2.5 mL twice daily for 2 months) showed an improvement in sperm concentration, motility, morphology and semen volume compared to placebo [43]. Interestingly, there was a negative impact of Ns oil on sperm viability in this study. Although this is generally contradictory to the current evidence available, there is a report of reduced motility with no negative effect on viability using an *in vitro* model on normozoospermic semen samples exposed to Ns extracts (5 and 10 mg/mL) [44].

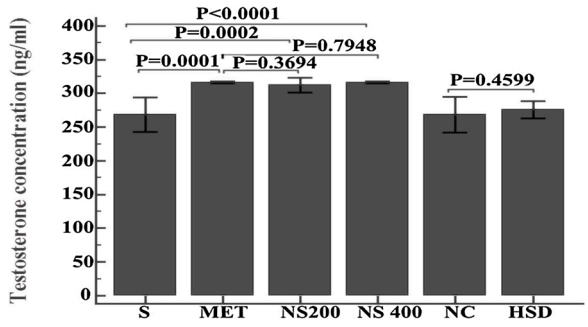

Fig. 8. Comparative analysis of the groups for testosterone concentration. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Although few Ns studies investigating obesity models and male reproduction were identified, there are reports of Ns on fertility parameters from healthy animal models. At 300 mg/Kg, an Ns seed aqueous extraction increased sperm concentration and motility within the cauda epididymis and testicular ducts, as well as increased numbers of spermatids and spermatocytes [45]. An ethanolic extract of Ns (200 mg/Kg) increased sperm concentration, testosterone and LH levels with no effect on FSH [46]. Similarly, Ns ethanolic extracts at higher dosages (500 and 1500 mg/Kg per day) increased testosterone and FSH, with a significant decrease in LH [47]. A high dose Ns oil extraction (1 mL/Kg) improved sperm concentration, motility and testosterone, and was reported to increase testicular antioxidants and reduce testicular reactive oxygen species [48]. At a lower dosage similar to the NS400 dosage in this study, 0.4 mL/Kg Ns oil improved sperm concentration with a decrease in lipid peroxidation and increased glutathione peroxidase in the testes, but had no effect on sperm motility or serum testosterone, inhibin, LH or FSH [49].

Ns, or the isolated compound thymoquinone, have been shown to protect against toxic damage in the testes. This is seemingly mediated primarily through improvement of oxidative stress in serum and in the testes. This includes protection against impaired sperm motility, normal morphology, viability, sperm membrane integrity, MMP and testosterone [48,50,51]. In a streptozotocin-induced diabetic rat model that induced testicular toxicity, Ns seed extractions significantly improved insulin concentration, testosterone, LH, FSH, dyslipidaemia, testicular redox balance (reduced malondialdehyde and increased SOD concentrations), and epididymal sperm concentration. These results were similar to the effect of metformin on these parameters, and were found to have a more positive outcome when metformin and Ns was combined [37,52,53].

The antioxidant activity of Ns oil may be due to various micronutrients and secondary plant metabolites. These include calcium (1860 mg/Kg), phosphorus (527 mg/Kg), iron (105 mg/Kg), niacin (57 mg/Kg), thiamine (15.4 mg/Kg), copper (18 mg/Kg), zinc (60 mg/Kg) and folic acid (160 μg/Kg) [7]. The oils are predominantly comprised of linoleic (50–60%), oleic (20–23%) and palmitic acids (12.5 %), alongside stearic, myristic and arachidonic acids, β-sitosterol, sterol esters and sterol glucosides [8]. Well-studied active constituents and antioxidants are isolated as aromatics from the essential oil of the seeds, particularly thymoquinone, dithymoquinone, thymol, carvacrol, α-pinene, β-pinene and α-thujene. Important alkaloids isolated from the seed oils include nigellicine and nigellidine. Other secondary plant metabolites with antioxidant activity include saponins, flavonoids, cardiac glycosides and alkaloids [7,8].

The improvement of metformin on body weight and testosterone was statistically similar to that of NS200 and NS400, as was the positive impact of metformin and NS400 on sperm MMP. However, metformin had a more pronounced positive impact on sperm concentration compared to NS200 and NS400. There was no statistical difference between metformin and NS200 or NS400 groups for sperm progressive motility and viability, where the metformin group had a statistically higher non-progressive motility compared to NS200, but not NS400. These results suggest the impact of metformin and Ns oil in this study were generally very similar in terms of parameters improved. Furthermore, for total body weight, all experimental exposures were similar to that of the normal chow group, and metformin and NS400 improved sperm MMP and serum testosterone better than NC group. These results suggest that metformin and Ns interventions may restore the negative impact of HSD to normal for these parameters.

The results of this study in the context of the current literature suggests that both metformin and Ns may be beneficial in male reproductive dysfunction induced by obesity. The mechanisms, however, remain generally elusive. Metformin has numerous cellular effects that explain its action on hyperglycaemia and insulin resistance syndrome [54]. Suggested mechanisms include a reduction of oxidative stress through increased endogenous antioxidant regulation, immune regulation through a reduction in inflammatory cytokines and M_1 macrophage expression, mild inhibition of mitochondrial respiratory chain complex 1 associated with reduced hepatic gluconeogenesis while increasing glucose utilisation peripherally, and activation of the 5′-AMP activated protein kinase (AMPK) pathway [13,15,54–57]. Metformin reduces both inflammation and associated oxidative stress through direct immune modulation, and indirectly through improvement of hyperglycaemia, insulin resistance and dyslipidaemia mediated by AMPK activation and NFκB inhibition [58,59]. In diabetes, metformin is shown to improve testicular inflammation and oxidative stress [60].

Similarly to metformin, Ns has also been established to modulate immune regulation, reduce oxidative stress and activate the AMPK pathway [13,61,62]. Ns seed oil and isolated thymoquinone activate $PPAR_{\Upsilon}$ and GLUT4 mRNA expression, upregulates antioxidant activity, improves insulin regulation and hyperglycaemia in streptozotocin-induced diabetic rats [62]. Ethanolic extracts of Ns have shown $PPAR_{\Upsilon}$ agonist activity through AMPK activation in an *in vivo* model on skeletal muscle and hepatocytes, improving insulin signalling pathways [63]. Alkaloid isolates from Ns seeds increase glucose absorption in hepatocytes through activation of AMPK, specifically nigelanoid, nigeglanine, nigellidine, 17-O-(β-D-glucopyr-anosyl)-4-O-methylnigellidine, 4-O-methylnigellidine and 4-O-methylnigeglanine [64]. For thymoquinone, an immune regulating effect through the activation of AMPK pathways, downregulation of TLR4 and inflammatory cytokine expression, as well as inhibition of phosphatidylinositol 3-kinase (PI3K) phosphorylation [65], has also been established. Sertoli cells further increase lactate production with exposure to Ns, with reduced GLUT1, GLUT3 and MCT4 expressions activated by AMPK in spermatozoa [15].

AMPK has important regulatory functions in cellular metabolism, proliferation and immune regulation, particularly relevant to the balance of glucose, lipids and protein metabolism [66]. Activation of AMPK results in GLUT4 translocation to the plasma membrane, increasing cellular uptake of glucose [66]. This pathway is activated by an increased AMP:ATP ratio, exercise and stress, and through exogenous compounds including polyphenols and synthetic molecules like metformin [67]. AMPK activation is known to result in a reduction of oxidative stress and may improve sperm parameters such as motility, morphology, and the acrosome reaction [15]. Metformin and Ns activation of the AMPK pathway results in inhibition of enzymes involved in gluconeogenesis and improving insulin signalling and intracellular glucose transport in muscles [13,61,62]. AMPK is considered a central regulator in testicular and spermatozoa function, connecting reproduction pathways with energy balance, and is further reported to have important roles in steroidogenesis and spermatogenesis [67]. Activation of AMPK pathway further improves MMP in mature mammalian spermatozoa, as reported in the metformin and NS400 groups [68]. AMPK further increases the proliferation of Leydig and Sertoli cells, leading to increased sperm concentration and testosterone production, respectively, again both observed in the metformin and NS400 groups [68,69].

Although motility was not significantly improved in this study, activation of AMPK pathway may partly explain the positive impact of metformin and Ns on the male reproductive system in diet induced obesity for sperm concentration, MMP and testosterone.

5. Conclusion

Ns oil and metformin were similarly effective in reducing total body weight, increasing sperm concentration, sperm MMP and serum testosterone levels in rats fed an obesogenic diet. Progressive motility and viability were not affected by Ns oil or metformin. Ns oil and metformin may offer benefit in male obesity with reproductive complications, potentially mediated through numerous potential pathways, including immune regulation, antioxidant activity and increased AMPK activity. Further clinical studies are warranted to investigate the mechanisms and therapeutic potential of these compounds in male obesity and associated infertility.

Source of funding

This work was supported by the Libyan Embassyin South Africa. They had no role in the study design, data collection, analysis and interpretation, in the writing of the report and in the decision to submit the article for publication.

Declaration of Competing Interest

The authors declare there are no conflicts of interest

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