A novel action of insulin sensitizing drug as a potential promotor of preovulatory follicles, ovulation rate and prolificacy in sheep: the first exploratory study

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Abstract

Impact of insulin-sensitizing drug metformin on preovulatory follicle (POF) turnover, ovulation rate, and prolificacy was investigated in forty-six cyclic Malpura ewes. Following estrus synchronization, the ewes were equally divided into two groups (n = 23). Treatment group (MET) received a daily oral dose of metformin at the rate of 500 mg/animal for around 12 wk, spanning five estrous cycles, as against untreated control (CON). All the ewes were bred with proven sires at the end of treatment. Ultrasonographic ovarian scans were carried out on every estrus and D 9 of each cycle to evaluate the number and diameter of POFs and corpora lutea (CL), respectively. A comprehensive assessment was conducted on circulating hormones including, estradiol, progesterone, androstenedione, and insulin as well as metabolic indicators like glucose, and lipid profile parameters. By the end of medication, the treatment showed a stimulatory effect on follicular turnover with a 53.2% (P < 0.001) rise in the number of POFs. It further elevated the ovulation rate by 67.4% (P < 0.01), with a higher proportion (χ² df1 = 10.7, P < 0.001) of ewes in MET group having multiple ovulations compared to the CON (82.6 vs. 30.4%, respectively). The proportion of ewes delivering multiple lambs was 2.9-fold higher in MET group than the CON. The plasma estradiol, insulin, glucose, total cholesterol, and LDL-cholesterol concentrations were lower (P < 0.05) in the MET ewes than in the CON. The findings of the present study indicate that metformin can augment POF numbers, ovulation rate, and prolificacy in ewe concurrent with reduced plasma estradiol, insulin, glucose and cholesterols in MET ewes.

Introduction

Nutritional security becomes an emerging need and one of the deep concerns within the contemporary epoch with food scarcity consequent to expanding global populace. Striving to enhance the ovine prolificacy, our study aimed to investigate the effect of metformin on ovarian turnover of POFs and ovulation rate. The increasing demand for animal protein in diet has rendered modifications in production systems and breeding strategies for the food animals. There is growing interest in pharmacologic or nutritional means to complement the genetic potential for exploiting optimal reproductive efficiency and thereby harvesting maximum production. Sheep is a prolific breeder where the litter size often ranges from one to four lambs per breeding cycle in different ovine breeds. The economic returns in sheep farming are determined by the number of lambs born per ewe. Several factors affect the fecundity in this species, including ovulation rate, fertilization rate, embryonic losses, fetal development, still births and neonatal mortalities (Juengel 2018). Amongst, ovulation rate stands out as a key determinant of litter size, as it sets the upper limit on number of lambs being born. There has been a persistent interest among the scientific community and breeders towards prolificacy improvement in the ovine breeds that produce singletons in most instances. In this context, various approaches such as nutritional (Viñoles et al. 2005; Somchit-Assavacheep et al. 2013), hormonal and immunological (Robinson and Scaramuzzi 1986) have been attempted to enhance the prolificacy in sheep with variable success rates. Nevertheless, nutritional intervention like flushing has shown limited efficacy in augmenting prolificacy in ewes, especially those with a normal body condition (Stubbings 2007; Yıldırım et al. 2022). Similarly, exogenous hormones and
immunization also remained commercially ineffective means of inducing twinning in ewes (Webb et al. 1999).

Previously, ample scientific reports have indicated the involvement of insulin-glucose metabolism at ovarian level (Willis and Franks 1995; Poretsky et al. 1999; Williams et al. 2001; Nishimoto et al. 2006; Neganova et al. 2007) to influence the follicular growth and thereby ovulation rate and prolificacy outcomes. In support of these observations, recently we have also witnessed the pro-folliculogenic and pro-ovulatory effects of n-3 PUFA rich fish oil through dietary supplementation in ewes and the associated modulations in insulin-glucose metabolism and ovarian steroidogenesis (Mahla et al. 2023). Owing to insulin sensitizing property (Lombardo and Chicco 2006; Oner and Muderris 2013), the n-3 PUFA have drawn the attention of researchers in recent decades, to explore their effectiveness in treating polycystic ovary syndrome (PCOS) in women (Yuan et al. 2021). Concomitantly, the beneficial effects of metformin, a biguanide insulin sensitizing drug commonly used for type 2 diabetes treatment, have also been demonstrated in reproductive healthcare, particularly treatment of PCOS in women (Velazquez et al. 1997; Lord et al. 2003; Palomba et al. 2009).

The therapeutic benefits of metformin and n-PUFA in restoration of ovulatory dysfunction in PCOS and the associated comparable modulations in serum-biochemicals and ovarian steroids, suggest a common thread of mechanisms. Taken together, both n-3 PUFA and metformin enhance insulin sensitivity and thereby promote the peripheral glucose uptake, exert a protective effect on serum lipid profile, and share certain common molecular signalling pathways. For instance, n-3 PUFA (Motawi et al. 2010; Yuan et al. 2021) and metformin (Prabhakar and Doble 2009; Bai et al. 2019) both act in a peroxisome proliferator-activated receptors (PPAR) dependent manner and activate the 5′-AMP-activated protein kinase (AMPK) signalling pathway. Thus, taking into account all these findings collectively, we hypothesized that metformin, resembling in action to n-3 PUFA, may also promote preovulatory follicle (POF) development and ovulation rate in ewes. Therefore, the present study was carried out to investigate the effect of metformin administration on POF yield and ovulation rate in otherwise single fetus bearing Malpura sheep. Additionally, the study examined the effect of metformin on plasma levels of ovarian steroids, insulin and metabolites that regulate folliculogenesis.

**Materials and Methods**

**Experimental animals and maintenance**

The study was carried out on healthy, breedable Malpura ewes that were routinely vaccinated and dewormed. Forty-six cyclic Malpura ewes in their first to third parity, aged between 2 to 5 y, with a mean body wt of 41.95±0.64 kg and a mean body condition score (BCS) of 3.27±0.06 on a five-point scale (Kenyon et al. 2014), were included in the experiment. These animals were maintained in iso-managerial conditions and reared in semi-intensive system at ICAR- CSWRI, Avikanagar, Rajasthan (India), which is located in the semi-arid region of India. The institute is situated at Longitude 75°28´E, Latitude 26°26´N and Altitude 320 m above mean sea level. A uniform concentrate feed, containing maize-45%, barley-45%, groundnut cake-4%, mustard cake-3%, mineral mix-2% and table salt-1%, was offered in both the groups at
the rate 300 g per day per animal, in addition to 4 h grazing. All the experimental animals had a free access
to clean potable water. Prior to the experiment, all ewes were examined through ultrasonographic scanning
of ovaries and uterus to confirm reproductive soundness and ovarian cyclicity.

**Experimental design and drug-regimes**

The experimental ewes were synchronized for estrus prior to onset of medication to bring all the animals in
similar reproductive state by inserting intravaginal progesterone sponges (AVIKESIL-S®, ICAR-CSWRI,
Avikanagar) which were kept *in situ* for a period of 12 d. The estrus synchronized ewes (N = 46) were
assigned into two equal groups (n = 23 in each group) as treatment versus control. From the day of
synchronized estrus (E0D0), animals in treatment group (MET) received a dose of metformin at 500
mg/animal/day *per os* (Padmanabhan et al. 2015) for a duration of approximately 12 wk, which covered
five estrous cycles, while the untreated ewes served as control (CON).

**Detection of estrus and breeding**

Throughout the medication period, estrus detection was carried out in all the ewes involved, starting from
the time of sponge withdrawal and continuing during subsequent estrous cycles until breeding. For the said
purpose, aproned ram parading was exercised twice daily (at 0600 and 1800 h) combined with visual
observations for 30 min until cessation of estrus in every cycle. The ewes standing still and allowing the
ram to mount, were deliberated as being in estrus. At the end of medication period (E5D0), all the ewes
were mated with proven rams twice in estrus at 12 h interval. The ewes which returned to estrus within
three wk of mating, were mated again for the second time.

**Ultrasonographic scanning**

Ovarian scans were performed using ultrasonography (Chison Eco Vet 6; China) with linear array B-mode
real time endorectal transducer (R7A, 5-10 MHz) to evaluate the follicular and luteal attributes (size and
number) on D 0 (day of estrus) and D 9 of estrous cycles, respectively, for six consecutive cycles. Follicles
with 5 mm in diameter or larger on the day of estrus were considered POF. The number of corpora lutea
(CL) in each cycle reflected the ovulation rate. Confirmative diagnosis of pregnancy and an initial detection
of fetal number was done on D 30 post-breeding by transrectal ultrasonography. Re-examination with
transabdominal ultrasonography (Sonosite Micromaxx, convex probe, 3.5 to 5.0 MHz) was carried out on D
45 of gestation for further confirmation of fetal number.

**Blood sampling**

Blood sampling was done on D 0 and D 9 of each estrous cycle in the morning before feeding at 0800 h.
Blood samples were collected by jugular venipuncture using 20-ga hypodermic needle and sterilized test
tubes containing ethylenediaminetetraacetic acid (EDTA). The blood samples were subjected to
centrifugation at 1000 × g for 15 min to obtain plasma, which was preserved at -20°C for subsequent
biochemical and hormone analysis.
Analysis of plasma hormones and metabolites

The plasma concentrations of ovarian steroid hormones particularly, progesterone, estradiol and androstenedione were assayed by competitive radioimmunoassay (RIA) procedure using RIA kits (Immunotech, Prague, Czech Republic). Analytical sensitivity of progesterone detection was 0.04 ng/mL and coefficients of intra- and inter-assay variation were ≤ 9.48 and ≤ 16.85%, respectively. The same for detection of estradiol were 10.41 pg/mL, ≤ 10.0 and ≤ 16.4%, respectively, whereas for androstenedione were 0.04 ng/mL, ≤ 10.1 and ≤ 14.6%, respectively. The concentration of insulin in plasma was measured by Immunoradiometric assay (IRMA; Immunotech, Prague, Czech Republic). The analytical and functional sensitivities of the insulin kit were 0.49 µIU/mL and 1.35 µIU/mL, respectively, whereas the intra-assay coefficient of variation was ≤ 3.99%. The circulating concentrations of glucose and lipid profile parameters namely total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride were measured using commercial kits (Accurex Biomedical PVT. LTD., Mumbai, India). Low-density lipoprotein (LDL)-cholesterol was computed by the Friedewald’s equation [LDL=TC−(HDL+TG/5)]. The hormones, except progesterone, and metabolic indicators were measured in plasma samples of estrus, whereas progesterone was determined in the D 9 samples of every cycle. Two replicates were employed for each sample while measuring the plasma hormones and metabolites.

Statistical Analysis

Data analysis was done using GraphPad Prism software package for windows (Version 8.0; GraphPad Inc., San Diego, CA, USA). Adherence to the assumption of normal distribution of data was tested by Shapiro-Wilk test and verified through examination of skewness, kurtosis and Q-Q plot. Time series data such as number of POFs and CL, plasma hormones and biochemicals were analyzed using GLM repeated measure ANOVA or Mixed Model ANOVA depending on missing values, with terms for treatment, time and their interactions included in the model as factors. The effect of treatment on particular day was evaluated by multiple comparisons of the means between treatment versus control group using Bonferroni test as Post-hoc test. The data pertaining to fetal count on D 30, 45, and lambing was analyzed using an unpaired t-test. Yates’ continuity corrected chi-square test was applied to analyze the proportion of ewes with multiple POF, ovulation, and fetuses between the groups. Values of different variables are presented as mean ± SEM and their differences are deemed significant at $P < 0.05$, while tendency is drawn at $0.05 \leq P \leq 0.1$.

Results

Follicular attributes

The effects ($P < 0.001$) of treatment and time as well as their interaction were observed on the number of POFs (Fig. 1). The number of POF gradually increased in time-dependent manner and became distinctly elevated ($P < 0.05$) after two estrous cycles in the MET group as compared to the CON. By the end of medication, the number of POF was 53.2% higher ($P < 0.001$) in MET than the CON ewes (2.13 ± 0.11 vs. 1.39 ± 0.12). The Fisher’s exact test with Yate’s correction revealed that the proportion of the ewes having multiple POF at mating (E5D0) was markedly greater ($\chi^2_{df1} = 16.2, P < 0.001$) in the MET group than the
CON (95.7 vs. 34.8%, respectively). A treatment ($P < 0.001$) and time ($P < 0.01$) effects were observed on diameter of largest POF, which was smaller ($P = 0.08$) in the MET ewes than in the CON during the last two estruses.

**Luteal attributes**

A treatment effect ($P < 0.001$), time effect ($P < 0.01$) and their interaction ($P < 0.01$) were observed on CL number (Fig. 2). Corresponding to the number of POF, the ovulation rate also improved progressively in treated ewes which became discernible ($P < 0.05$) from E3 onwards and eventually exceeded to CON ewes by 67.4% ($P < 0.01$) during the last estrus ($2.26 \pm 0.12$ vs. $1.35 \pm 0.17$, respectively). During the last estrus, 82.6% of ewes in the MET group had multiple ovulations compared to only 30.4% in the CON ($\chi^2_{df1} = 10.7, P < 0.001$) (Fig. 3). No significant effect of treatment ($P = 0.07$) and time ($P = 0.57$) as well as their interaction ($P = 0.59$) was observed on the diameter of largest CL, which remained comparable between the groups across the duration of study.

**Conception rate, pregnancy rate, fetal count, and prolificacy**

Based on cyesiognosis through transrectal ultrasonography on D 30 of gestation, first service conception rate was recorded as 91.3 and 86.9% in MET and CON group, respectively; whereas, 100 and 86.9% ewes were pregnant after second mating of repeated ewes in respective groups (Table 1). The number of fetuses detected on D 30 of gestation (Fig. 4) was greater ($P = 0.02$) by 36.5% in MET ewes than the CON ($1.57 \pm 0.15$ vs. $1.15 \pm 0.08$). Subsequently on D 45 of gestation, even with some embryonic mortalities in MET ewes, the overall fetal rate remained higher ($P = 0.04$) than the CON ($1.43 \pm 0.11$ vs. $1.15 \pm 0.08$). The proportion of ewes carrying multiple fetuses on D 45 was 2.9-fold greater ($\chi^2_{df1} = 2.87, P = 0.09$) in MET than CON group (43.5 vs. 15.0%, respectively). During lambing, the MET group showed a higher occurrence of multiple births, with 43.5% of ewes delivering twins and triplets compared to only 15.0% in the CON ($\chi^2_{df1} = 2.87, P = 0.09$). Except for one fetus of triplet in a MET ewe which was missed during ultrasonography, the lambs born were in line with the fetal number detected on D 45 of gestation and the prolificacy was also higher ($P = 0.04$) in MET group than the CON ($1.48 \pm 0.12$ vs $1.15 \pm 0.08$).

**Table 1** Effect of metformin for 12 wk on conception rate, pregnancy rate, fetal number and prolificacy in ewes
### Table 1: Reproductive Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>First service conception rate (%)</td>
<td>86.9</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td>(20/23)</td>
<td>(21/23)</td>
</tr>
<tr>
<td>Overall conception rate (%)</td>
<td>76.9</td>
<td>92.0</td>
</tr>
<tr>
<td></td>
<td>(20/26)</td>
<td>(23/25)</td>
</tr>
<tr>
<td>Pregnancy rate (%)</td>
<td>86.9</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(20/23)</td>
<td>(23/23)</td>
</tr>
<tr>
<td>Fetal rate on D 30 of gestation</td>
<td>1.15±0.08 (23/20)</td>
<td>1.57±0.15 (36/23)</td>
</tr>
<tr>
<td>Fetal rate on D 45 of gestation</td>
<td>1.15±0.08 (23/20)</td>
<td>1.43±0.11 (33/23)</td>
</tr>
<tr>
<td>Prolificacy</td>
<td>1.15±0.08 (23/20)</td>
<td>1.48±0.12 (34/23)</td>
</tr>
</tbody>
</table>

Figures in the parenthesis indicate number of observations.

### Plasma Hormones

Effects of treatment ($P < 0.01$) and time ($P = 0.03$) along with their interaction ($P < 0.01$) were obtained on plasma concentration of estradiol (Fig. 5a). Following the commencement of treatment, estradiol concentration declined gradually and remained lesser ($P < 0.05$) from E3 onwards in MET ewes than the control. The plasma concentration of progesterone measured on D 9 did not differ significantly between the groups across the study (Fig. 5b). Similarly, androstenedione concentration also remained comparable between the groups (Fig. 5c). An effect of treatment ($P = 0.04$), time ($P = 0.06$) and their interaction ($P = 0.04$) was seen on circulating insulin concentration (Fig. 5d). Plasma insulin decreased gradually in treated ewes and the difference in insulin level between the groups attained significance in E4 ($P = 0.02$) and E5 ($P < 0.01$).

### Plasma Metabolites

An effect of treatment ($P < 0.01$) and time ($P < 0.001$) was noted on plasma glucose concentration which decreased ($P < 0.05$) towards the end of medication in treated ewes as compared to control ones (Fig. 6a). In lipid profile, a time effect ($P = 0.04$) was observed on total cholesterol and post-hoc analysis showed that its concentration was lesser ($P = 0.04$) in MET ewes than their control counterparts at the end of treatment (Fig. 6b). A treatment ($P = 0.07$) and time ($P = 0.05$) effect along with their interaction ($P = 0.07$) was observed on plasma LDL-cholesterol concentration. It showed a reducing trend and decreased ($P < 0.01$) during E5 in the treated ewes as compared to the CON (Fig. 6c). No treatment effect ($P > 0.05$) and its
interaction with time ($P > 0.05$) was observed on HDL-cholesterol (Fig. 6d) as well as triglycerides (Fig. 6e) concentrations.

**Discussion**

There is a linkage between reproduction and the physiological mechanisms that control energy homeostasis, wherein metabolites, hormonal mediators, and modulators of energy balance influence the reproduction also. In a study to explore the effect of dietary n-3 PUFA on key molecules involved in modulation of folliculogenesis, we observed that mRNA expressions of PPAR-γ, a metabolic sensor that is largely involved in the follicle development, and GLUT-4, an insulin-dependent potent glucose transporter, were significantly upregulated (data unpublished), with improved insulin sensitivity and decreased estradiol production in fish oil supplemented ewes (Mahla et al. 2023). The fact that metformin improves ovarian functions in rat (di Pietro et al. 2015; Mahamed et al. 2018) and human (Pirwany et al. 1999; Shpakov 2021) through enhancing peripheral insulin sensitivity with improved glucose transportation (Ismail et al. 2015; Heber et al. 2019) and altering ovarian steroidogenesis (Mansfield et al. 2003; Faure et al. 2018) in a synergistic action with PPAR system (Pan et al. 2009; Prabhakar and Doble 2009; Bai et al. 2019), encouraged us to study the effect of metformin on follicular development and ovulation rate in ewes. Metformin is a well-tolerated medication with no reported detrimental effect on physiological processes (Aljada and Mousa 2012). Despite this, its potential role in enhancing reproductive performance in female livestock animals has not been evaluated, so far. To date, investigations into the benefits of metformin in animal reproduction remain limited, with only a small number of studies examining its effects on sperm kinematics in boar (Li et al. 2020), dog (Grandhaye et al. 2020), and buck (Zhao et al. 2022). Though, we have not observed any untoward effect of metformin, yet the data are still insufficient to completely confirm or disprove negative effects of metformin.

Findings of the present study indicated that metformin potentiates the follicular turnover as witnessed by a substantial increase (53.2%) in the number of POF with 2.75 times more ewes having multiple POF on their ovaries in the MET group as compared to the CON. These results are in agreement with a previous study in androgenized rat, where authors found that metformin restored the follicular dynamics with an increase in the number of ovulatory follicles and a significant decrease in steroidogenesis by suppressing the proliferation of theca cells and CYP-17 expression (Mahamed et al. 2018).

It is known that intra-follicular insulin-glucose system facilitates the stimulatory response of energy supplementation to increase the follicle turnover and ovulation rate in small ruminants (Scaramuzzi et al. 2006). Hence, the pro-folliculogenic effect of metformin may be attributed to an increased insulin sensitivity and thereby enhanced glucose uptake (Bailey and Turner 1996) and improvement in insulin dependent growth factors at follicular level (Stadtmueller et al. 2001; di Pietro et al. 2015; Mahamed et al. 2018). The improvement in follicular development following metformin administration in this study was associated with a simultaneous decrease in the systemic levels of insulin similar to the earlier reports in the PCOS rats, where, metformin treatment lowered the insulin level and restored follicular dynamics (Tjwa et al. 2003; di Pietro et al. 2015). Besides, clinical studies in PCOS affected patients have revealed that metformin improves IGF-1 level along with a decrease in IGFBP-1 in the follicular fluid of preovulatory
follicles and thereby increases the number of mature oocytes, ovulation rate, fertilization rate and pregnancy rate (Stadtmauer et al. 2001). Supplementary to this, improvement in follicular development, with an increase in number of antral follicles and CL after metformin administration in PCOS rats has been attributed to VEGF upregulation causing ovarian angiogenesis and neovascularization (di Pietro et al. 2015; Mahamed et al. 2018). Hence, it can be postulated that the observed enhancement in follicular dynamics in treated ewes may be attributed to the increased insulin action in the ovaries induced by metformin. This hypothesis is reinforced by previous reports demonstrating the advantageous effects of exogenous insulin administration on folliculogenesis in various animal species, including doe (Selvaraju et al. 2003), gilts (Matamoros et al. 1991), cattle (Simpson et al. 1994) and mice (Godaneh et al. 2023).

The increased POF number in our study was consistent with a significantly higher ovulation rate as evident by 67.4% hike in the same in treated group with a larger proportion of ewes displaying multiple ovulations after treatment (82.6 vs. 30.4%, respectively). Unexpectedly, the ovulation rate turned out to be higher than the number of POFs recorded, particularly in the MET ewes. This could be attributed to the likelihood that the metformin triggered the ovulation from the medium sized follicles approaching 5 mm diameter, cut-off criterion used to classify a follicle as preovulatory in the study. Abundant clinical observations and scientific reports have established that metformin elicit ovulatory response through systemic and local effects exerted by a variety of mechanisms viz. reducing insulin levels and altering ovarian steroidogenesis and theca cell proliferation (Elnashar 2011). Metformin being AMPK (AMP-activated protein kinase) activator, can regulate ovulation centrally by modulating GnRH release through activation of the hypothalamic AMPK (Coyral-Castel et al. 2008). The essential involvement of AMPK in the regulation of folliculogenesis, steroidogenesis, and meiotic activity in oocyte that control its maturation (Tosca et al. 2007; Shpakov 2021), further endorse the metformin's local effect on folliculogenesis and ovulation rate. This notion is reinforced again with the findings of a study in knocked out mice, wherein deletion of AMPK α1-subunit in the oocytes led to reductions in litter size by 27 and 68% following in vivo and in vitro fertilization, respectively (Bertoldo et al. 2014).

The significant reduction in the diameter of the largest POFs in metformin treated ewes was associated with a concurrent decline in plasma insulin and cholesterol levels, consistent with our previous study in ewes (Mahla et al. 2023). Previous studies have shown that the altered microenvironment of the follicle resulting from high concentrations of insulin (Zachut et al. 2008) and cholesterol (Renaville et al. 2010) in fat-supplemented cows increases granulosa cell proliferation, leading to larger ovulatory follicles. A similar increase in ovulatory follicle diameter has been observed in cows fed a high-fat diet (Lucy et al. 1991; Thomas and Williams 1996), which has been attributed to either improved energy balance or the direct impact of lipid components on developing ovarian follicles (Staples et al. 1998). Contrarily, metformin has been reported to inhibit granulosa cell proliferation (Tosca et al. 2006, 2007). The smaller diameter of POFs in MET ewes could be attributed to the fact that the follicles with a higher count of POFs have fewer granulosa cells, leading to smaller follicle size (Shackell et al. 1993).

Relatively more ewes became pregnant in metformin treated group (100%) as against untreated controls (86.9%), though non-significantly ($P = 0.23$). In a few studies, treatment of PCOS women with metformin had shown to improve pregnancy rates and live birth rates (Batukan and Baysal 2001; Tang et al. 2012;
A significantly greater fetal rate obtained in MET group is a desired and applied outcome of this study which encourages for further research to re-validate the results and to explore the potential of this drug as an adjunct to fecundity improvement in the small ruminants. A significant improvement in developmental competence of porcine oocytes and embryos was observed when in vitro production culture was exposed to metformin and insulin combinedly (Lee et al. 2005). A slightly higher prolificacy than the fetal rate detected on D 45 of gestation in treated ewes was attributed to a triplet birth in one ewe from the MET group, which was misdiagnosed as twin during ultrasound assessments. The observed significant enhancement in fetal rate and prolificacy among MET ewes compared to CON ewes was definitely an outcome of increased ovulation rate. Furthermore, the favourable impact of metformin on oocyte and embryo developmental competence may have also played a role in the improved reproductive performance of treated ewes.

Considering the tight linkage between reproductive outcomes and endocrine-metabolic milieu, assessment of circulating hormones namely estradiol, progesterone, androstenedione, and insulin along with glucose and lipid profile parameters was carried out. A significant reduction in plasma estradiol concentration in our study is concurrent to previous studies, where reduction in estradiol concentration was observed as a result of direct suppressive action of metformin on aromatase (CYP19A1) activity through AMPK signalling pathway in human (la Marca et al. 2000, 2002; Mansfield et al. 2003) and bovine (Tosca et al. 2007) granulosa cells. In addition, metformin has also been found to decrease the abundance of 3b-HSD, StAR, CYP11A1, and CYP19A1 proteins, while inhibiting granulosa cell proliferation in rat (Tosca et al. 2006) and bovine cell lines (Tosca et al. 2007). Reduced circulating oestradiol concentration during follicular phase concurrent to a declined aromatase activity in granulosa cells, after infusion of glucose (Gallet et al. 2011) or lupin grain supplementation (Kosior-Korzecka and Bobowiec 2003; Somchit-Assavacheep et al. 2013) has shown to improve ovulation rate in ewes. Similarly, improved folliculogenesis and ovulation rate have also been reported to be associated with reduced plasma estradiol concentration following n-3 PUFA supplementation through fish oil in does (Mahla et al. 2017) and ewes (Mahla et al. 2023). The low plasma estradiol is supposed to increase the cohort size of gonadotrophin-responsive follicles by exerting a less suppressive effect on FSH, and therefore, more follicles grow further and reach ovulatory size (Scaramuzzi et al. 2011). This might be one of the possible mechanisms through which metformin stimulates the number of POFs and thereby ovulation rate as observed in this study.

It has been shown that metformin decreases basal or FSH-stimulated progesterone and estradiol concentrations in PCOS women and in cultured granulosa cells from women with or without PCOS (Nestler and Jakubowicz 1996; la Marca et al. 2002; Mansfield et al. 2003). However, in the present study, the plasma progesterone concentration did not differ significantly between the groups throughout the course of treatment. This observation corroborates to previous similar studies attempting fecundity improvement in does (Mahla et al. 2017) and ewes (Mahla et al. 2023), where increased prolificacy in fish oil supplemented animals was associated with a significant decrease in plasma estradiol whereas, no significant alteration in circulating progesterone. The authors suggested that reduced cholesterol substrate linked steroidogenesis in fish oil supplemented animals was compensated by reduced progesterone.
clearance associated with increased PPARs (Galbreath et al. 2008). In this study, it is possible that metformin inhibited progesterone clearance by acting as an agonist to PPAR-γ (Tosca et al. 2006).

Plasma androstenedione concentration in the current study remained comparable between the groups. Our results are in line with previous reports citing no significant change in androstenedione levels after metformin administration in human (Acbay and Gundogdu 1996; Nestler and Jakubowicz 1996; Ehrmann et al. 1997; La Marca et al. 2000; Moghetti et al. 2000) and mice (Roland and Moenter 2011).

A significant decrease in plasma insulin concentration in metformin treated ewes in our study corresponds to most of previous studies in rat and human, wherein plasma insulin concentration was reduced as a result of enhanced peripheral sensitivity to insulin and its action in peripheral tissues (Bailey and Turner 1996; Giannarelli et al. 2003; Ismail et al. 2015; Heber et al. 2019). Moreover, a significant reduction in the plasma glucose of metformin treated ewes might have predisposed the fall in circulating insulin, as a result of feedforward control (Aljada and Mousa 2012). Accumulating evidences have shown that metformin-mediated improvements in insulin sensitivity may be associated with several mechanisms, like increased activity of insulin receptor tyrosine kinase, enhanced glycogen synthesis, and an increased recruitment and activity of GLUT4 glucose transporters. Additionally, metformin promotes the re-esterification of free fatty acids and inhibits lipolysis in adipocytes, which may further improve insulin sensitivity indirectly through reduced lipotoxicity (Giannarelli et al. 2003; Ismail et al. 2015; Heber et al. 2019). The evidence of insulin's ability to modulate steroidogenesis in cultured ovarian cells (Barbieri et al. 1983) and the identification of insulin receptors in both stromal and follicular compartments of the ovary (Poretsky et al. 1984, 1985) have recognized the ovary as another important target organ for insulin action. Moreover, the reduced plasma insulin due to improved insulin sensitivity in association with altered steroidogenesis in this study supports the hypothesis of insulin mediated modulation in steroidogenic enzymes (Diamanti-Kandarakis and Papavassiliou 2006).

The glucose-lowering effect of metformin observed in this study coincides with many published reports in human and rodents (Stumvoll et al. 1995; Bailey and Turner 1996; Cheng et al. 2006; Scarpello and Howlett 2008) and is attributable to both an increased peripheral glucose uptake and a decreased glucose production in liver (Hundal et al. 2000; Hunter et al. 2018). The peripheral glucose uptake is induced by insulin and involves glucose transporters (GLUTs). GLUT-1 is located predominantly in the plasma membrane, whereas GLUT-4 is stored in storage vesicles in the basal state, but upon insulin stimulation it is translocated to the plasma membrane, inducing glucose uptake (Szymczak-Pajor et al. 2022). Metformin has been demonstrated to stimulate the translocation of GLUT-4 from the intracellular space to the cell surface via AMPK-dependent regulation in pre-adipocyte cells (Lee et al., 2012). Interestingly, GLUT1 and GLUT4 have been identified in granulosa and theca cells from sheep (Williams et al. 2001) and cattle (Nishimoto et al. 2006). GLUT4 is an insulin-dependent potent glucose transporter having a high capacity of glucose transportation and hence usually expressed in tissues where there is a huge demand for glucose like skeletal and cardiac muscles or adipose tissue. The intra-follicular presence of GLUT4 enlightens the covert role of insulin and the insulin mediated glucose uptake in the follicle (Scaramuzzi et al. 2010). The action of metformin is mainly anti-hyperglycaemic, and it doesn't cause any clinical hypoglycaemia (Batukan and Baysal 2001). Our study also confirmed this, as the fasting glucose concentration remained
within the physiological range (Reid 1950; McCann et al. 1986), even with a significant reduction in MET ewes.

It has been postulated that stimulation of AMPK by metformin, confers insulin sensitivity, mainly by modulating lipid metabolism (Pernicova and Korbonits 2014). Hence, we next evaluated the plasma lipid profile and noted a significant reduction in total cholesterol and LDL-cholesterol in MET group compared to their control counterparts. The suppressive effect of metformin on plasma cholesterol and LDL concentrations observed in this study is in line with several previous findings in humans (Robinson et al. 1998; Solymár et al. 2018) and rats (Lu et al. 2013). The mechanism behind this reduction in plasma LDL and total cholesterol levels in metformin-treated individuals may be attributed to the activation of AMPK signalling pathway, which results in an increase in fat oxidation and a decrease in lipogenesis (Woo et al. 2014). The activation of AMPK by metformin leads to phosphorylation of acetyl-CoA carboxylase (ACC), leading to a decrease in malonyl-CoA levels and an increase in carnitine palmitoyltransferase-1 activity, ultimately promoting mitochondrial fatty acid oxidation. Furthermore, AMPK inhibits the expression of lipogenic genes like fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and ACC in the liver, thereby reducing lipid storage (Malin and Kashyap 2014). This reduced cholesterol level in circulation may further defend the decrease in estradiol concentration observed in present study, since the steroidogenesis requires an active delivery of the cholesterol as a precursor substrate (Stocco and Clark 1996). In addition to decreasing the systemic cholesterol, metformin also down-regulates the expression of StAR, an important cholesterol carrier (Tosca et al. 2007).

In conclusion, the oral administration of metformin for about 12 weeks significantly improved the number of POF, ovulation rate and fetal rate in ewes. Simultaneously, the metformin administration also exerted a significant suppressive effect on circulating concentrations of estradiol, insulin, total cholesterol and LDL-cholesterol. The findings stemmed out of this study, unveil a new insight on metformin's implication in prolificacy improvement in small ruminants, which warrants further detailed studies to underpin the exact molecular mechanisms by which metformin improves follicular development and ovulation rate in ewes.

**Declarations**

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**Credit author statement** B.L. Kumawat as doctoral student performed investigation, data curation, formal analysis; validation and wrote the original draft. Pramod Kumar as major advisor supervised the study, reviewed and edited the draft. A.S. Mahla as research guide conceived the idea, provided the resources, administered the project, did formal analysis and improved the original draft. Ashok Kumar helped in methodology and formal analysis. Amit Kumar further reviewed and edited the draft. Raghvendar Singh as project administrator managed the resources and laboratory facilities. Arun Kumar acquired funding and provided animal resources.
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Animal Ethics approval and consent  This research trial was approved by the Institutional Animal Ethics Committee (IAEC) vide approval No. IAEC/353/2016-17/05 and all experimental procedures were performed according to the guidelines of the ethics committee, ICAR- Central Sheep and Wool Research Institute (CSWRI), Avikanagar, Tonk, Rajasthan, India.

Competing interest  None of the authors has any personal or financial conflict of interest to declare.

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Figures
POF turnover (a) and diameter of largest POF (b) assessed by transrectal ultrasonography in six consecutive estruses (E0 = onset of treatment; E5 = end of treatment) in ewes treated with metformin (MET; n=23) for a period of 12 wk as against the control (CON; n=23). Representative ovarian sonogram of ewes showing multiple POFs (c) as indicated by vesicle diameter 1 & 2. A time-dependent stimulatory effect ($P < 0.001$) of metformin treatment was observed on POF number. An effect of treatment ($P < 0.001$) and time ($P < 0.01$) on the diameter of the largest POF was observed, with no significant interaction effect ($P = 0.61$). Significant differences between groups were determined by Bonferroni Post-hoc test. Data are shown as mean ± S.E.M. POF: Preovulatory follicles. Single asterisk: $P < 0.05$; Triple asterisks: $P < 0.001$
Ovulation rate (a) and diameter of largest CL (b) assessed by transrectal ultrasonography on day 9 of six consecutive estrous cycles (E0 = onset of treatment; E5 = end of treatment) in ewes treated with metformin (MET; n=23) for a period of 12 wk as against the control (CON; n=23). Representative ovarian sonograms of ewes with two or more CL indicating multiple ovulations (c, d). A treatment effect ($P < 0.001$), time effect ($P < 0.01$) and their interaction ($P < 0.01$) were observed on CL number. The diameter of largest CL remained comparable between the groups across the days of treatment without any significant effect of treatment, time and their interaction. Significant differences between groups were determined by Bonferroni Post-hoc test. Data are shown as mean ± S.E.M. CL: Corpus luteum. Single asterisk: $P < 0.05$; Triple asterisks: $P < 0.001$
Figure 3

Doughnut split analysis representing the effect of metformin on proportion of ewes with multiple ovulations during the treatment span. The proportion of ewes (percentage) with multiple ovulations increased significantly ($\chi^2_{df1} = 10.7, P < 0.001$) from E0 to E5 in treated ewes (MET) as compared to control (CON).
Figure 4

Representative transrectal ultrasonogram of uterus showing twin fetuses on D 30 of gestation in ewes administrated with oral metformin
Figure 5

Effect of metformin treatment on plasma concentration of (a) estradiol (pg/mL), (b) progesterone (ng/mL), (c) androstenedione (ng/mL), and (d) insulin (µIU/mL) in ewes. Significant differences between groups were determined by Bonferroni Post-hoc test. Data are shown as mean ± S.E.M. A significant decrease ($P < 0.01$) in plasma estradiol and insulin was seen in treated ewes following the metformin treatment for 12 weeks. Single asterisk: $P < 0.05$; Double asterisks: $P < 0.01$; Triple asterisks: $P < 0.001$
Figure 6

Effect of metformin treatment on plasma glucose (a) and lipid profile parameters including total cholesterol (b), LDL-cholesterol (c), HDL-cholesterol (d), and triglycerides (e) in ewes. Significant differences between groups were determined by Bonferroni Post-hoc test. Data are shown as mean ± S.E.M. Metformin exerted a significantly reducing effect on glucose ($P < 0.01$) and LDL-cholesterol ($P < 0.01$). Single asterisk: $P < 0.05$; Double asterisks: $P < 0.01$