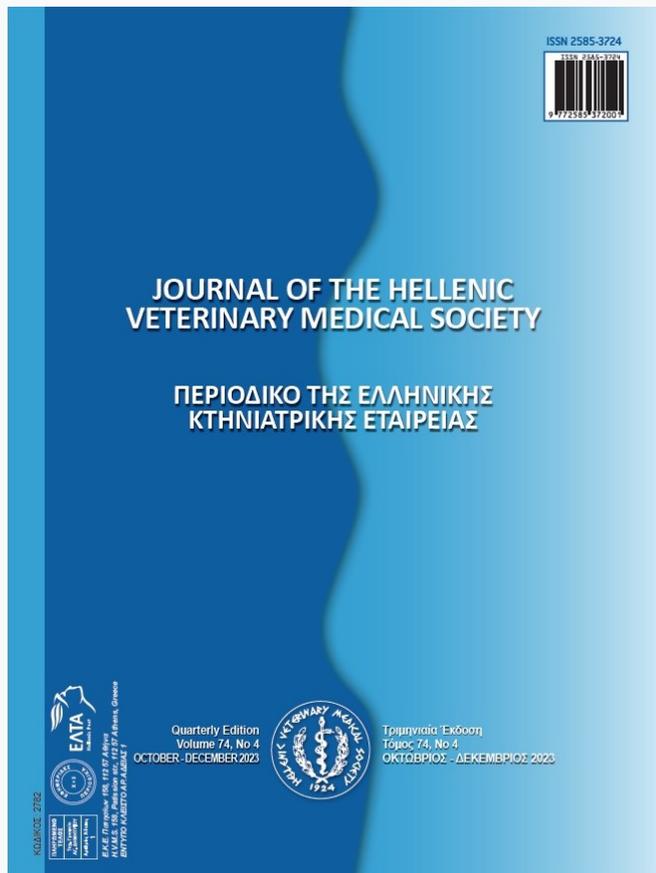


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## Stimulation of inflammatory response in chicken Sertoli cells following metformin exposure

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**ABSTRACT :** The testis is considered as an immune-privileged site, and testicular Sertoli cells have been identified as key players for conferring this immune privilege. These cells play an important physiological role in the testis, as they support, nourish and protect the germ cells, as they present immune properties such as immune factors secretion, phagocytosis and constitute also the blood testicular barrier which limit damage to germ cells. The aim of the present study was to evaluate the inflammatory response in chicken Sertoli cells induced by metformin, an insulin-sensitizer drug which also possess anti-inflammatory properties, by studying the expression pattern of innate immune-related genes, including Toll-like receptors (TLRs), and cytokines, after a kinetic stimulation (0 to 48 hours) by metabolic dysregulation metformin induced. Chicken Sertoli cells were exposed to lipopolysaccharide (1µg/ml, as LPS used to mimic a bacterial infection) or metformin. Metformin stimulated expression of some Toll Like Receptors (TLR1-2; 2-1), but did not modify expression of the TLR4, known to be bound by LPS, as well as the increase in expression of some interleukins (IL-1β, IL-6, IL-8, IFN-γ) after metformin stimulation and even more after an LPS and metformin exposure. In conclusion, modification of energy cell metabolism by metformin lead to activation of some set of immune system in the chicken Sertoli cells and even more after activation by bacterial component (LPS).

**Keywords:** Sertoli cells; metformin; innate immunity; Toll-like receptors

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## INTRODUCTION

As rooster infertility is a major concern in the poultry industry, protection of the male reproductive organs from pathogens is an essential aspect of reproductive physiology. Testis is constituted from Sertoli cells, Leydig cells, peritubular cells and germ cells. The testis is considered an immune-privileged site and testicular Sertoli cells (SCs) have been identified as key players for conferring this immune privilege (Selawry 1994, Dufour et al. 2003). SCs constitute the blood testicular barrier and establish tight junctions which limit germ cells damage and protect germ cells development in to spermatozoa in order to maintain fertility.

Sertoli cells present immune properties and are considered as macrophage like-like cells having phagocytosis functions, immune factors secretion and consequently can modulate immune response for preserved fertility (Pineau et al., 1991; Kaur et al 2014). It is now well established that the innate immune system utilizes Toll-like receptors (TLRs) to recognize and bind pathogen-associated molecular patterns (PAMPs), such as the bacterial endotoxin lipopolysaccharide (LPS), which is present in the cell wall of Gram-negative bacteria and acts as a well-established PAMP to the innate immune system, as it mimics many inflammatory effects (Akira et al., 2001). This binding of PAMPs leads to TLR activation, which initiates MAPK or nuclear factor kappa B (NFkB)-dependent cascades that culminate in a proinflammatory response. This response involves the secretion of cytokines, which are essential for the activation, differentiation, and control of the immune system and also play a key role in initiating innate and adaptive immune responses (Kogut et al., 2006).

Previous studies have reported that mouse Sertoli cells are able to express thirteen tolllike receptors (TLR) after activation of their TLR signaling pathways (Wu et al., 2008). We have previously reported that 9 members of the chicken TLR family were expressed in the chicken testis, while *Salmonella enteritidis* (SE) infection resulted in a significant induction of certain TLR genes in the testis of sexually mature birds, compared to healthy birds of the same age (Anastasiadou et al., 2011). Furthermore, we have also reported that several cytokines, as well as TLRs were expressed in the rooster reproductive tract, while SE infection resulted in a significant up-regulation of many cytokines and TLR genes of SE-infected roosters (Anastasiadou and Michailidis 2016). Moreover,

lipopolysaccharide (LPS) injection resulted in up-regulation of TLRs expression after 6h and pro-inflammatory cytokines after 3h and 6h (Zhang et al., 2012), while Sertoli cells isolated from rat in primary culture produced some cytokines, particular interleukins IL-1 and IL-6 regulated by testosterone level, which plays an important role in proliferation and differentiation of germ cells (Stephan et al., 1997) attest to Sertoli cells importance to preserve fertility.

Chronic inflammation signaling pathways are related with metabolic disorders such as obesity and type 2 diabetes and several studies have associated the potential role of inflammation process between the obesity, angiogenesis, cancer prevalence and infertility, associated with a significant decrease in the motility and the number of spermatozoa per ejaculate and oligozoospermia (González et al., 2015; Morrison et al., 2015; Xie et al., 2016; Fukumura et al., 2016). Several insulin sensitizer drugs used in treatment of type 2 diabetes, present the anti-inflammatory and antioxidant properties, which are desirable for autoimmune disease therapy (Tomczynska et al., 2016). Among them, metformin, is also described as an energy restriction mimetic agent, which has the ability to block the respiratory chain in mitochondria, modify the AMP/ATP ratio by decreasing ATP production, increasing lactate formation and activating the AMP-activated protein kinase (AMPK) (Rena et al., 2013). Metformin, which is an insulin sensitiser, is known to mimic food restriction and may modulate male fertility and interestingly, AMPK activated in mice stimulates also the antiinflammatory process through the suppression of TLR4 activity (Vaez et al., 2016). In chickens, metformin induces metabolism changes in the testis, and lead to a reduction in the testis growth and a delay spermatogenesis, but the relationship between metabolism and the reproductive immunity is not clearly know activity (Vaez et al., 2016).

As chickens are naturally hyperglycaemic (2 g/L) and are relatively insulin resistant, many studies have use them as a model for studying type 2 diabetes in humans or obesity (Walzem et al., 2014). The aim of the present study was therefore to investigate if metabolic disturbances induced by metformin contribute also to changes in the inflammatory response in chicken Sertoli cells, in order to evaluate the interactions between cell metabolism and immune system in the testis.

## MATERIALS AND METHODS

### Isolation of chicken Sertoli cells and seminiferous tubules

For each culture, cells were purified from testis of 12-weeks-old roosters (Rhode Island Reds), as previously described (Anastasiadou et al., 2011). Sertoli cells were cultured in HEPES-buffered F12/Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma), 5 % Foetal Calf Serum (FCS) in a humidified atmosphere of 5 % CO<sub>2</sub> at 37°C. Cells were seeded at 5×10<sup>5</sup> cells/well (in six well plate). After the first 24h of culture, the medium was changed to DMEM/F12 without FCS for 24h, then cells were exposed or not with metformin (5 mM, 1,1dimethylbiguanide hydrochloride, Sigma) or LPS (1µg/ml) from *Escherichia Coli* Serotype O111:B4 (Sigma), for 6, 12, 24 or 48h to mimic inflammation or both substances for 48h. Media and cells were collected and stored at -80°C until analysis. 6 different cultures were performed, as described in figure legends.

### RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from Sertoli cells stored at -80°C. The RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion), according to the instructions provided by the manufacturer. The preliminary quantity and purity of the extracted RNA was measured at 260 and 280 nm using the BioPhotometer (Eppendorf) and RNA integrity was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. To reduce degradation, RNase inhibitor (Invitrogen) was added to each sample (1 Unit per µg of RNA) before storage at -80°C. All samples were pretreated, before reverse transcrip-

tion (RT), with DNase (Fermentas) at a concentration of 1 Unit per µg of RNA. One µg of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer instructions.

Quantitative expression analysis of TLR and cytokines genes was performed with real-time PCR, using a LightCycler real-time PCR machine (Roche Molecular Biochemicals), as previously described (Michailidis et al., 2010), using the primers illustrated in Table 1. PCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems) and 0.2 pmol of each primer in a final volume of 20 µl using as template 1/10 of the initial cDNA synthesis reaction. Gene expression levels were quantified using the β-actin as an internal standard for cDNA normalization. The cycling parameters were: incubation at 95°C for 10 min, followed by 45 cycles of incubation at 95°C for 10 sec, 56-59°C (Table 1) for 8 sec, 72°C for 8 sec, read at 60°C. For identification of the PCR products a melting curve was performed from 65 to 95°C with read every 0.2°C and 5 sec hold between reads. All the reactions were performed six times using cDNA synthesized from RNA extracted from different cell cultures. The threshold cycle (Ct) values of the PCRs were averaged and relative quantification of the transcript levels was performed using the comparative Ct method (Livak and Schmittgen 2001). Real-time PCR data were analyzed using the 2<sup>-ΔΔCt</sup> method (Livak and Schmittgen 2001) to calculate the relative level of each mRNA in each sample and expressed as a ratio relative to β-actin housekeeping gene. The expression levels of both metformin and metformin + LPS cell cultures are indicated as relative values of untreated control cells, in order to compare the differences in the expression levels in the treated groups.

**Table 1:** Primer sequences of the chicken cytokine and TLRs genes used for real-time PCR.

Gene	Forward	Reverse	Annealing Temp. (°C)
IL-1β	GCATCAAGGGCTACAAGCTC	CAGGCGGTAGAAGATGAAGC	56
IL-6	CTCCTCGCCAATCTGAAGTC	GGATTGTGCCCGAACTAAAA	56
IL-8	GATTGAACTCCGATGCCAGT	TCCACATTCTTGCAAGTGAGG	59
IFN-γ	GACCGCACATCAAACACATA	TCCTTTTGAAACTCGGAGGA	56
TLR1-1	TTACTGCCAATTGCTTGAC	GGTTAGGAAGACCGTGTCCA	56
TLR1-2	CCCGTTCAAGTGTTTCATGTG	GTTCCGCTCAAGTCTTCTGG	56
TLR2-1	ACATGTGTGAATGGCCTGAA	TTGAGAAATGGCAGTTGCAG	56
TLR2-2	TCTCAACACGCTGAGGATTG	CAGCTGTTGCTGCAATCCTA	56
TLR3	CCCTGATGGAGTGTGTTGCTT	CAGCTGTTGCTGCAATCCTA	59
TLR4	GTTTGACATTGCTCGGTCCT	GCTGCCTCCAGAAGATATGC	56
β-actin	CTCCCTGATGGTCAGGTCAT	ATGCCAGGGTACATTGTGGT	56

## Statistical analysis

The significance of differences among the means of the experimental groups of cells was examined using one-way ANOVA, followed by Tukey's multiple range test for *post-hoc* analysis. The Levene's test for Homogeneity of Variances was applied in order to test the assumption that groups have approximately equal variance on the dependent variable. The results of the *post-hoc* analysis determine which of the groups are the most statistically different. Furthermore, a t-test was performed to estimate the significance of differences among the Sertoli cells in all experimental groups. Results were expressed as the mean  $\pm$  SEM. In all applications (one-way ANOVA and t-tests) differences were considered statistically significant at  $P < 0.05$ .

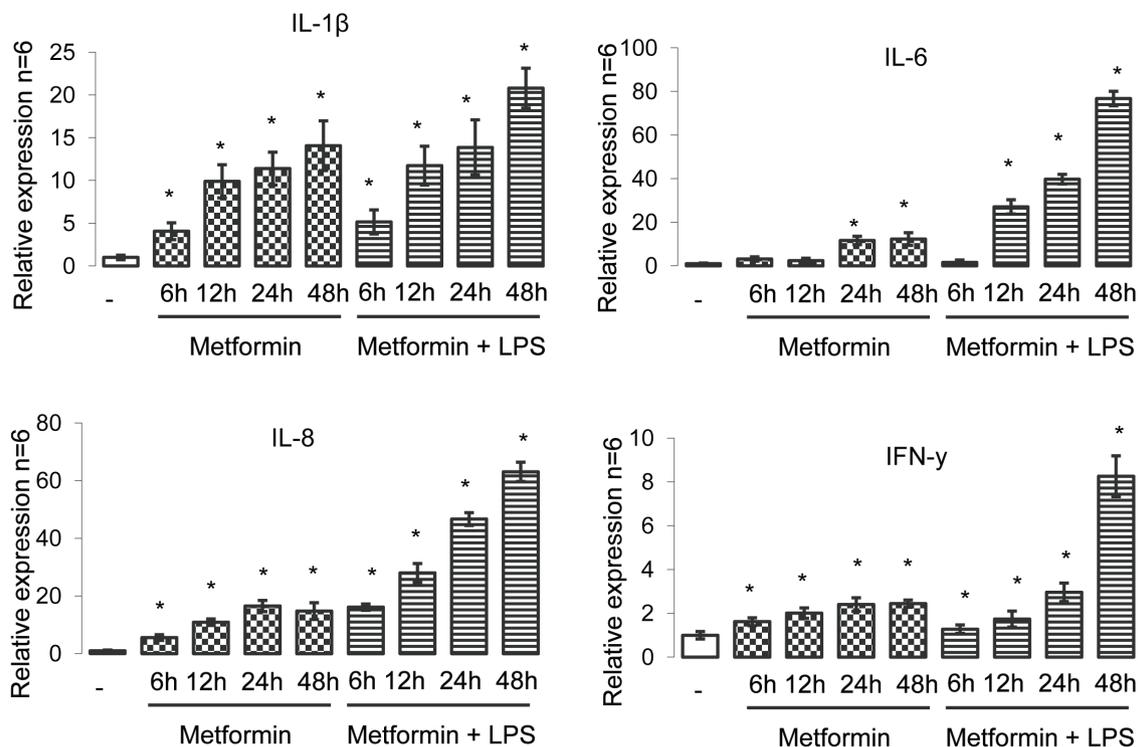
## RESULTS

In the following experiments we have collected innate immune response from chicken Sertoli cells after modulation of cell metabolism by metformin *in vitro*. Chicken Sertoli cells were exposed for 6, 12, 24 or 48h with metformin (5mM) in order to induce a metabolic disturbance, and also cells were treated for 6, 12, 24 or 48h with metformin and LPS to induce an inflammatory reaction. After 6, 12, 24 or 48h

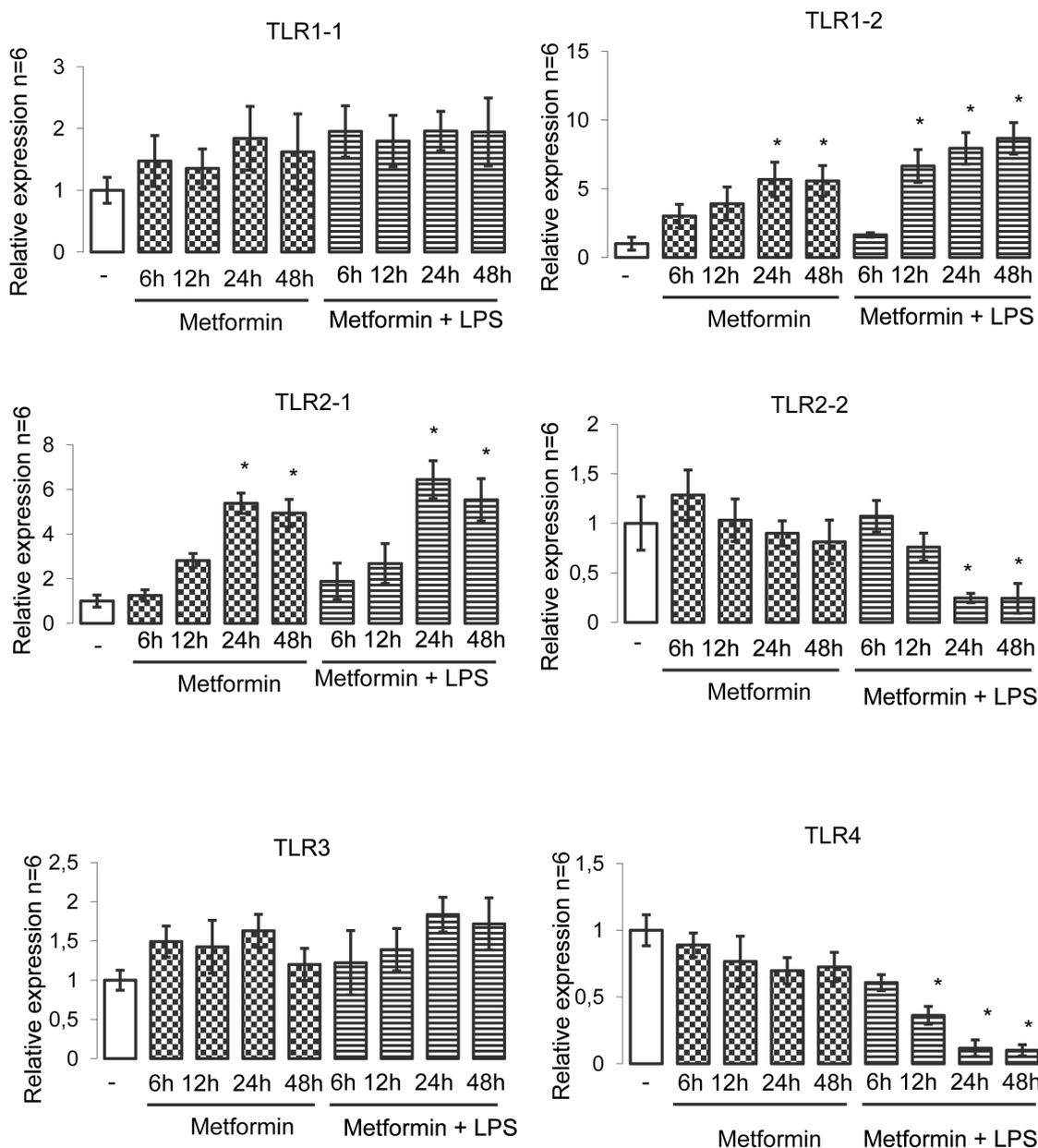
with metformin and with metformin and LPS, RNA was extracted from these cells and was analysed by real-time PCR for the mRNA changes in the expression levels of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and IFN- $\gamma$  and the TLRs 1-1, 1-2, 2-1, 2-2, 3 and 4.

Stimulation of chicken Sertoli cells by metformin for 6-48 hours resulted in a significant ( $P < 0.05$ ) up-regulation of the expression levels of the pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8 and IFN- $\gamma$ ) (Fig. 1), while this up-regulation was significantly higher after co-stimulation of metformin and LPS.

Furthermore, quantitative real-time PCR analysis revealed that the treatment of cells with metformin and with metformin and LPS resulted in significant ( $P < 0.05$ ) upregulation in the expression levels of TLR1-2 and TLR2-1 after 24 hours of metformin stimulation and LPS exposure after a metformin pre-stimulation compared to unstimulated condition (Fig. 2), while no significant changes ( $P > 0.05$ ) in the expression levels of TLR1-1, TLR2-2, TLR3 and TLR4 genes were observed after metformin and metformin and LPS stimulation for 6, 12, 24 or 48h respectively (Fig. 2).



**Fig. 1.** Changes in the expression of cytokines IL-1 $\beta$ , IL-6, IL-8 and INF- $\gamma$  in chicken primary Sertoli cells treated with metformin (5mM) for 0, 6, 12, 24 and 48 hours or pretreated with metformin and stimulated by LPS (1  $\mu$ g/ml) for 0, 6, 12, 24 and 48 hours. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean  $\pm$  SEM (n=6). Asterisk indicates that the difference in the expression levels were significantly different ( $P < 0.05$ ).



**Fig. 2.** Changes in the expression of TLR genes TLR1-1, TLR1-2, TLR2-1, TLR2-2, TLR3 and TLR4, in chicken primary Sertoli cells treated with metformin (5mM) for 0, 6, 12, 24 and 48 hours or pretreated with metformin and stimulated by LPS (1  $\mu$ g/ml) for 0, 6, 12, 24 and 48 hours. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean  $\pm$  SEM (n=6). Asterisk indicates that the difference in the expression levels were significantly different (P<0.05)

## DISCUSSION

In the present study we aimed to investigate the anti-inflammatory effects of metformin in relation to its regulatory role on inflammatory responses and to investigate whether metabolic disturbances induced by metformin could result in an inflammatory response in chicken Sertoli cells, by analyzing the changes in the expression levels of key genes of the chicken innate immune mechanism, such as the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and IFN- $\gamma$  and members of the chicken TLR family, such as TLRs 1-1, 1-2, 2-1, 2-2, 3 and 4. Previous studies have reported

the immunological role of Sertoli cells in the innate immune mechanisms of the testis and in the initiation of testicular immune responses (Riccioli et al., 2006; Starace et al., 2008; Wu et al., 2008; Sun et al. 2010). As the knowledge of the innate immune gene function is an emerging aspect in the chicken industry in order to control infectious diseases, we investigated the time course of an inflammatory response in chicken SCs triggered by exposure to metformin and the bacterial LPS, which is present in the bacterial membrane wall and acts as a well-established PAMP to the innate immune system. Metformin, an

insulin sensitiser from the biguanide family of molecules, is a drug used for the treatment of type 2 diabetes and is associated with anti-inflammatory reaction, but the underlying mechanism is unclear. Metformin has also been associated with increased ovulation, fertilization, and pregnancy rates, normalization of the endocrine profile, as well as a return to normal menstrual cyclicity (Weaver and Ramachandran, 2023). Based on the tight link between metabolism and fertility, we investigated the effect of metformin on the inflammatory response in chicken Sertoli cells induced, by lipopolysaccharide. Exposure of Sertoli cells by LPS pre-treated with metformin was a model to evaluate the interaction between cell metabolism and immune system in the testis. As the interaction of TLRs and their ligands induces cellular responses (including synthesis of proinflammatory cytokines and antimicrobial peptides), we used this *in vitro* model to investigate cytokine and TLR signaling in rooster SCs, in order to evaluate the interactions between cell metabolism and immune system in the testis.

Data on expression analysis presented in this study revealed that the expression levels of all the cytokines examined, including IL-1 $\beta$ , IL-6, IL-8 and IFN- $\gamma$  was significantly up-regulated following metformin and metformin + LPS exposure, especially after 48 hours of stimulation.

Previous studies have reported that IL-1 $\beta$ , IL-6 and IL-8 are mediators of inflammation. These cytokines are induced directly either by LPS or other cytokines and *in vitro* stimulation by mediators such as IL-1 $\beta$  or LPS in rodent SCs in turn stimulates integrin ligand expression and lymphocyte adhesion by SCs (Gerard et al. 1992, Riccioli et al. 1995). Furthermore, IL-8 has been shown to increase the metabolism of reactive oxygen species and can stimulate IL-1 $\beta$  expression. Expression of these cytokines, has also have been associated with the activation of Toll-like receptors pathways (El-Zayat et al., 2019). The expression analysis data, performed in this study, revealed that the expression levels of TLR1-2 and TLR 2-1 were significantly upregulated by metformin and LPS stimulation and that expression of TLR4 was diminished about 20%, after 48h of metformin stimulation, but not significantly, while metformin and LPS stimulation resulted in significantly down-regulation of TLR4. In rat cardiac tissue, metformin significantly decreased the high mRNA levels of TLR4 (Soraya et al., 2012) and in culture of murine neutrophils, the TLR4-induced activation was also reduced by the AMPK ac-

tivators (AICAR, berberine) and diminished the pulmonary injury (Zhao et al., 2008). Activation of the immune activity and cytokine secretion is associated to the TLR pathway in immune cells such as murine Sertoli cells (Wu et al., 2008). For example, LPS recognized as a pathogen by TLR4, induced a nuclear translocation of NF- $\kappa$ B and expression and secretion of pro-inflammatory cytokines (O'Neill et al 2013). In rodent neutrophil or kidney, metformin and AMPK activator increased the phagocytosis and autophagy activity suggesting that AMPK activation has the properties to induce bacterial eradication (Park et al 2013; Zhang et al., 2014). In addition, the use of metformin on LPS-stimulated rat microglial cell cultures did not impair the production of pro-inflammatory cytokines except IL-1 $\beta$  (Labuzek et al 2010). Metformin increased the LPS-induced production of IL-6, IL-10 and TGF-beta by microglia. The species and type of cells is a strong background leading to a stimulating or inhibiting consequences on cytokines production. Indeed, the AMPK activator AICAR inhibited the expression of proinflammatory cytokines induced by LPS (IL1 $\beta$ , IL6, TNF $\alpha$ ) in primary rat astrocytes, microglia, peritoneal macrophages and in bronchoalveolar tissue (Giri et al 2004, Zhao et al 2008). Absence of AMPK $\alpha$ 1 in mice increased inflammation and at the opposite, the use of A-769662, a specific activator of AMPK inhibited the IL-6 expression at both mRNA and protein levels, the phosphorylation of p65 NF- $\kappa$ B and MAPK phosphorylation (Guma et al 2015).

The data presented in this study are in accordance with several previously published studies, which have shown that LPS stimulation resulted in the up-regulation of several innate immune-related genes in various vertebrate species (Herath et al. 2006, Ibeagha-Awemu et al. 2008, Yunhe et al. 2013). Our results strongly suggest that in chicken, as in various mammalian species several molecular mechanisms involved in immune response are conserved and are functional in Sertoli cells and these data expand previous understanding and provide novel insight into the function of TLRs cytokines in SCs, as well as on metformin and LPS.

## CONCLUSIONS

Collectively, this study provides evidence on a possible mechanism by which metformin or cell metabolism changes stimulate pro-inflammatory process, such as upregulation of cytokine and TLR expression in chicken Sertoli cells. These data suggest that in chicken Sertoli cells modulation of cell metabolism

have to be taken into account in the regulation of the innate immune system. Future studies are necessary in order to evaluate the molecular and physiological mechanisms underlying the beneficial effects of metformin in improving the reproductive efficiency of

broiler breeder hens.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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