Received: 22 December 2020 | Accepted: 9 February 2021

DOI: 10.1111/aji.13401

ORIGINAL ARTICLE

AJRI American Journal of Reproductive Immunology WILEY

Transient systemic inflammation in adult male mice results in underweight progeny

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Funding information
Ministry of Education, Government of India, Department of Biotechnology, Government of India, Department of Science and Technology, Government of India.

Abstract

Problem: While the testes represent an *immune-privileged* organ, there is evidence that systemic inflammation is accompanied by local inflammatory responses. We therefore examined whether transient systemic inflammation caused any inflammatory and functional consequences in murine testes.

Method of Study: Using a single systemic administration of Toll-like receptor (TLR) agonists [lipopolysaccharide (LPS) or peptidoglycan (PG) or polyinosinic-polycytidylic acid (polyIC)] in young adult male mice, we assessed testicular immune-inflammatory landscape and reproductive functionality.

Results: Our findings demonstrated a significant induction of testicular TNF-α, IL-1β and IL-6 transcripts within 24 h of TLR agonist injection. By day 6, these cytokine levels returned to baseline. While there was no change in caudal sperm counts at early time points, eight weeks later, twofold decrease in sperm count and reduced testicular testosterone levels were evident. When these mice were subjected to mating studies, no differences in mating efficiencies or litter sizes were observed compared with controls. Nonetheless, the neonatal weights of progeny from LPS/PG/polyIC-treated sires were significantly lower than controls. Postnatal weight gain up to three weeks was also slower in the progeny of LPS/polyIC-treated sires. Placental weights at 17.5 days post-coitum were significantly lower in females mated to LPS- and polyIC-treated males. Given this likelihood of an epigenetic effect, we found lower testicular levels of histone methyltransferase enzyme, mixed-lineage leukaemia-1, in mice given LPS/PG/polyIC 8 weeks earlier.

Conclusion: Exposure to transient systemic inflammation leads to transient local inflammation in the testes, with persistent sperm-mediated consequences for foetal development.

KEYWORDS
epigenetics, inflammation, male fertility, placenta, progeny, sperm

Satyajit Rath and Vineeta Bal contributed equally.

1 | INTRODUCTION

As the primary site for the production of spermatozoa and testosterone, mammalian testes are crucial determinants of male fertility.[1] Further, unlike oogenesis in the female, testicular spermatogenesis is a continuous process.[2] As a result, changes in the testicular tissue microenvironment carry a significant potential for altering spermatozoal programming, with transgenerational consequences.[3] The blood-testis barrier and the resulting immune-privileged status of the testes become interesting in this context as well, although they are more commonly invoked from the perspective of prevention of autoimmune responses.[4,5]

Testicular inflammatory responses, both autoreactive and antimicrobial, particularly antiviral, are well-documented factors in male infertility.[6] A number of viruses show testicular tropism and initiate antiviral immune responses in the testis leading to inflammation, tissue damage and impaired testosterone and sperm production.[7-10] Systemic inflammatory states mediated by microbial ligands for pattern recognition receptors (PRRs) are known to induce testicular responses as well, although many studies connect such local responses to the maintenance of immune privilege.[11-13] Testicular cells express a broad array of PRRs such as Toll-like receptors (TLRs) and RIG-I-like receptors.[14-17] Using animal models of systemic administration of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, several studies have reported local testicular production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and of reactive oxygen species. Such inflammatory responses have been associated with short-term detrimental effects on testicular functions of spermatogenesis and steroidogenesis, breach of blood-testis barrier, induction of germ cell apoptosis and loss of sperm motility leading to infertility.[18-21]

However, there have been few studies examining whether transient systemic inflammation, that induces a similarly transient response in the testes, has any long-term functional consequences on reproductive potential. It has been reported that a single dose of systemic LPS administration can cause damage lasting up to 5 weeks to the testicular structure in mice with significant decrease in spermatogenesis, meiotic index and epithelial height.[22] Another recent study has similarly reported month-long testicular damage upon exposure to a single systemic LPS dose, with apoptosis of spermatogonial germ cells, severely impaired spermatogenesis, thickening of smooth muscle layers, infiltration of immune cells in the interstitial spaces and presence of germ cells in the lumen of cauda epididymis.[23] Such effects would be particularly relevant in communities with high microbial exposure loads, where mild-to-moderate systemic inflammation, commonly asymptomatic or only mildly symptomatic, would be a matter of common occurrence.

On this background, we report an analysis of the long-term functional consequences of transient systemic inflammation in the mouse testes. We report that a single systemic exposure of young adult male mice to TLR ligands showed transient local induction of pro-inflammatory cytokines, followed by a return to baseline within six days. However, sperm counts showed persistent mild reduction. When these male mice were used in mating studies, while they show unimpaired fertility, their progeny were born with significantly lower birthweights and showed persistent postnatal delays in weight gain. This functional epigenetic effect was accompanied by persistent reduction in the testicular levels of a histone methyltransferase, MLL1. These data open up mechanistic possibilities for inflammation-mediated epigenetic changes in the spermatozoal differentiation programme. Further, they open up translational possibilities as a factor for explaining the persistently high frequency of India's low birthweight problem.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6 male mice (aged 8 weeks) and C57BL/6 female mice with proven fertility (aged 12–14 weeks) were obtained from the National Facility for Gene Function in Health and Disease (NFGFHD), IISER, Pune. The study was reviewed and approved by the Institutional Animal Ethics Committee (IISER/IAEC/2018-01/05) and was conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)-approved guidelines. Animals were maintained under controlled conditions of temperature and humidity with 12-h light and 12-h dark daily cycle, and were fed ad libitum.

For inducing systemic inflammation, 8-week-old male mice were given TLR ligands namely LPS (5 mg/kg; from *Salmonella typhosa*, Sigma), or PG (5 mg/kg; from *Staphylococcus aureus*, Sigma), or polyIC (10 mg/kg; Sigma) by intraperitoneal (i.p.) injection. Control mice were similarly given normal saline. Animals were euthanised at 24, 72 and 144 h, or 8 weeks later. Body weights were recorded at each time point prior to euthanasia. Mice were anaesthetised with isoflurane, and blood samples were collected via the retro-orbital sinus for serum separation. Subsequently, cardiac perfusion was carried out with ice-cold PBS. Testes, spleen and brain tissues of perfused animals were collected in TRIsoln reagent (Genei) for RNA extraction and cytokine analysis, while cauda epididymides were collected in PBS for measuring the sperm count. Testes from the 8-week groups were frozen in liquid nitrogen for testosterone assays.

2.2 | RNA isolation and quantitative reverse transcriptase-PCR analysis

Total RNA extracted as above was treated with DNase I (Thermo Scientific) to eliminate any potential genomic DNA contamination. The yield and quality of the resulting RNA were evaluated by determining the A260:A280 ratio. RNA (700 ng) was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad). The cDNA obtained was used for subsequent real-time quantitative qPCR reactions (CFX96 Thermal Cycler, Bio-Rad). Reaction specificities were validated by melt curve analyses. For all experiments, 18S rRNA was used as internal control.

Primer sequences used for the amplification of cDNAs were designed using NCBI Primer BLAST Software (Supplementary Data, Table S1).

2.3 | Sperm count

Cauda epididymides from euthanised mice were collected in PBS. Incisions were made in the caudae to release the sperm, which were allowed to exude at 37°C for 30 min. The resulting sperm suspension was washed, resuspended and counted using a haemocytometer. Sperm counts were performed in replicates for each sample at two dilutions.

2.4 | Testosterone assay

Frozen testes were homogenised in 1 ml of ice-cold 1% SDS containing 0.5 N NaOH and incubated at 40°C for 3 h on a rotary shaker at 100 rpm. The homogenate was centrifuged at 13 000 g for 20 min at 4°C. The resulting testicular supernatants and serum samples were appropriately diluted, and testosterone levels were analysed using a testosterone-specific competitive ELISA Kit according to manufacturer's protocol (Diagnostic Biochem Canada Inc).

2.5 | Fertility studies

To study the effect of transient systemic inflammatory stress on fertility, 8-week-old male mice were given a single injection of LPS, PG or polyIC (or PBS as a control) as above. Eight weeks later, the reproductive capacity of these mice was evaluated by cohabitation with healthy syngeneic females. Briefly, each male was caged individually with two females of proven fertility for a maximum period of 20 days. Fertility assessment was done using the proportions of females achieving pregnancy, duration between the beginning of mating and delivery of the pups, and numbers of offspring per litter. Progeny evaluation was done using pup weights from birth up to 3 weeks of age. At 8 weeks of age, blood glucose levels were determined, and progeny mice were euthanised to determine body lengths and lean weights.

For assessing placental weights, mating studies were set up as described above, and females were examined daily for the presence of vaginal plugs to indicate successful mating. The day on which vaginal plugs were observed was considered as 0.5 dpc (day post-coitum). At 17.5 dpc, pregnant dams were euthanised and placental and foetal weights were recorded.

2.6 | Serum glucose assays

Blood was drawn retro-orbitally, and sera were separated. Glucose levels were estimated using an automated glucose analyser (DiaLab, GmbH, A-2351).

2.7 | Statistical analyses

Data were analysed using Prism software (Graph Pad). Student's *t* test was used to determine *p* values, and $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | A single systemic exposure to LPS-induced transient local testicular inflammatory responses and dysfunction

To test the testicular consequences of transient systemic inflammation, young male mice were given a single i.p. dose of LPS,[20] which is a component of the outer membrane of Gram-negative bacteria and a TLR4 agonist. Within 24 h, the mice showed symptoms of ruffled body hair, reduced locomotor and exploration activity (Supplementary methods; Figures S1-S2) and decreased body weight (Figure 1A). In concordance with these findings, local expression of a number of inflammation-related cytokine genes such as TNF-α, IL-1β and IL-6 was detectable, not only in the spleen (Figure S3), but also in immune-privileged sites such as the testes (Figure 1B-F) and the brain (Figure S4). However, these effects were transient; they began to decline by 72 h, and by 144 h after LPS administration, most parameters were essentially back to baseline levels (Figure 1; Figures S3-S4).

In association with these transient effects, the caudal sperm counts were significantly reduced in LPS-treated mice at 24 h. However, by 72 h, sperm counts had returned to normal levels in these mice (Figure 1G).

3.2 | Transient systemic exposure to TLR2 and TLR3 ligands, PG and polyIC, induced a milder testicular inflammation phenotype than that induced by LPS

Since transient exposure to a TLR4 ligand, LPS, induced short-lived local inflammation in the mouse testes, we next examined whether ligands for other TLRs also induced similar alterations. Based on preliminary titrations, we used doses of PG and polyIC that triggered detectable early systemic inflammatory responses. Young male mice were given single i.p. doses of either the TLR2 agonist, PG or the TLR3 agonist, polyIC. Unlike LPS, neither PG nor polyIC induced any transient behavioural alterations (Figures S5-S8). PG induced no transient weight loss either, although polyIC did (Figures 2A, 3A). However, as seen with the spleen (Figure S3) PG induced transient local testicular expression of some, though not all, inflammation-related cytokine genes tested (Figure 2B-2F). While polyIC challenge did not induce any inflammatory response in the spleen within 24 h (Figure S3), testis showed an induction of IL-1β, IL-6, IFN-γ and IL-10 transcripts (Figure 3B-3G). However, no such expression

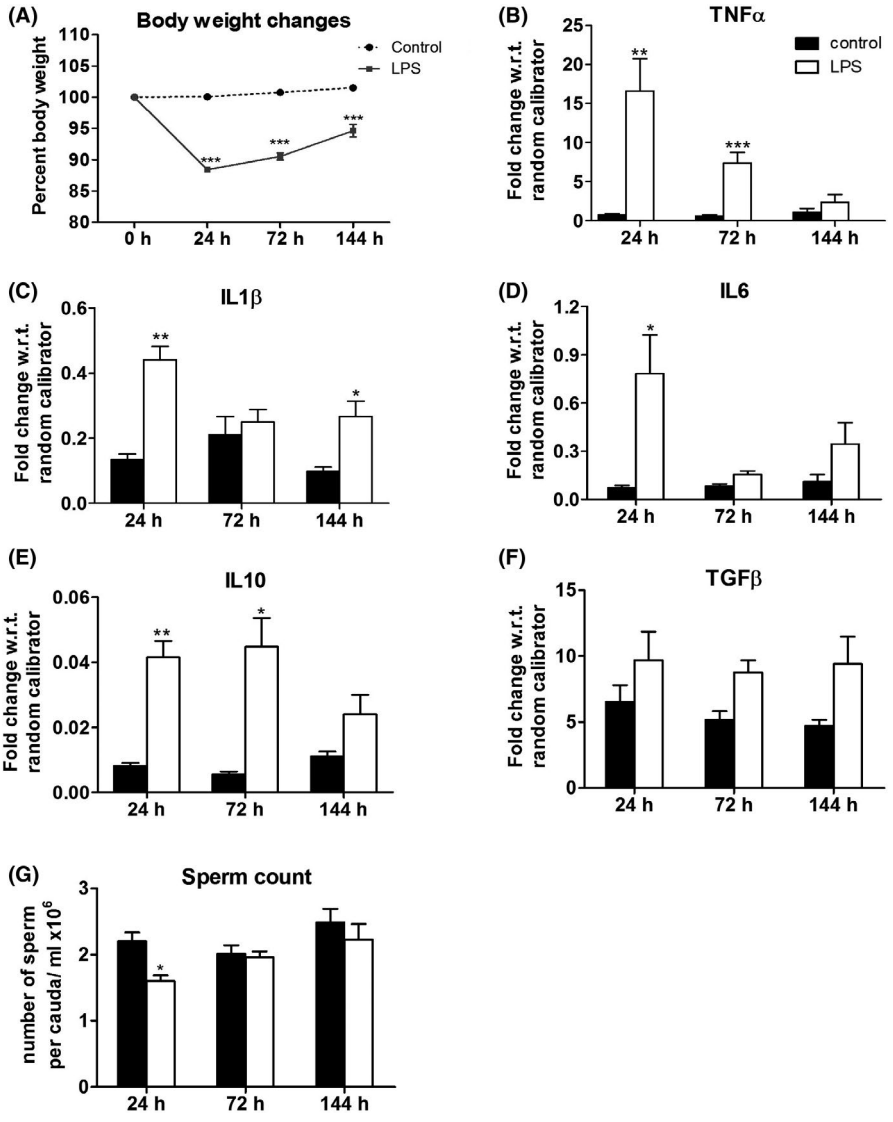

FIGURE 1 Effects of systemic lipopolysaccharide (LPS) injection on body weight, testicular cytokine milieu and caudal sperm counts in mice. Male mice were treated with LPS as described, and (A) body weight changes are shown as normalised to the weight at the beginning of the experiment (n = 9–31), and (B-F) testicular cytokine gene mRNA levels (n = 5) were assayed at various times as shown. Levels of mRNA for TNF-α (B), IL-1β (C), IL-6 (D), IL-10 (E) and TGF-β (F) were measured by qRT-PCR, and normalised values as shown were calculated. (G) Caudal sperm counts at indicated times post-LPS injection (n = 5–11). Data are shown as mean ±SE. * $p < .05$, ** $p < .01$, *** $p < .0001$

was reliably detected in the brains of these mice (Figures S9-S10). However, as with LPS treatment, all the detectable effects were transient; by 144 h after TLR ligand administration, most parameters were essentially back to baseline levels (Figures 2, 3). Unlike after LPS treatment, the caudal sperm counts were unaltered at 24–144 h in PG/ polyIC-treated mice (Figures 2G, 3H).

3.3 | Testicular dysfunction at 8 weeks post-LPS/ PG/polyIC exposure

It was evident from the data above that challenging young adult mice with a single systemic LPS dose induced only transient inflammatory responses in the testes, which were resolved by 144 h. We next tested whether this LPS-mediated transient local testicular inflammation had any long-term functional consequences in the testes. Since the early inflammatory phenotype was milder in mice given PG or polyIC, we used those ligands to test whether long-term consequences, if any, were similarly milder.

Interestingly, LPS-exposed male mice showed a twofold reduction in caudal sperm counts 8 weeks after LPS exposure, and this was true in PG/polyIC-treated mice as well (Figure 4A). Intratesticular testosterone levels were significantly lower in LPS- and polyIC-treated mice, but not in PG-treated mice (Figure 4B). While serum testosterone showed a tendency to lower levels, the differences were not significant (Figure 4C). Notably, there were no significant differences in the levels of TNF-α, IL-6, IL-10 or TGF-β in the testes of LPS/PG/polyIC-treated mice compared with saline controls, 8 weeks after exposure (Figure 4D, 4F-H). However, there was a marginal increase in IL-1β levels in PG- and polyIC-treated mice (Figure 4E).

3.4 | Effects of a single LPS/PG/polyIC exposure on progeny sired 8 weeks later

We next tested whether these features of testicular dysfunction affected the fertility potential of LPS/PG/polyIC-exposed male

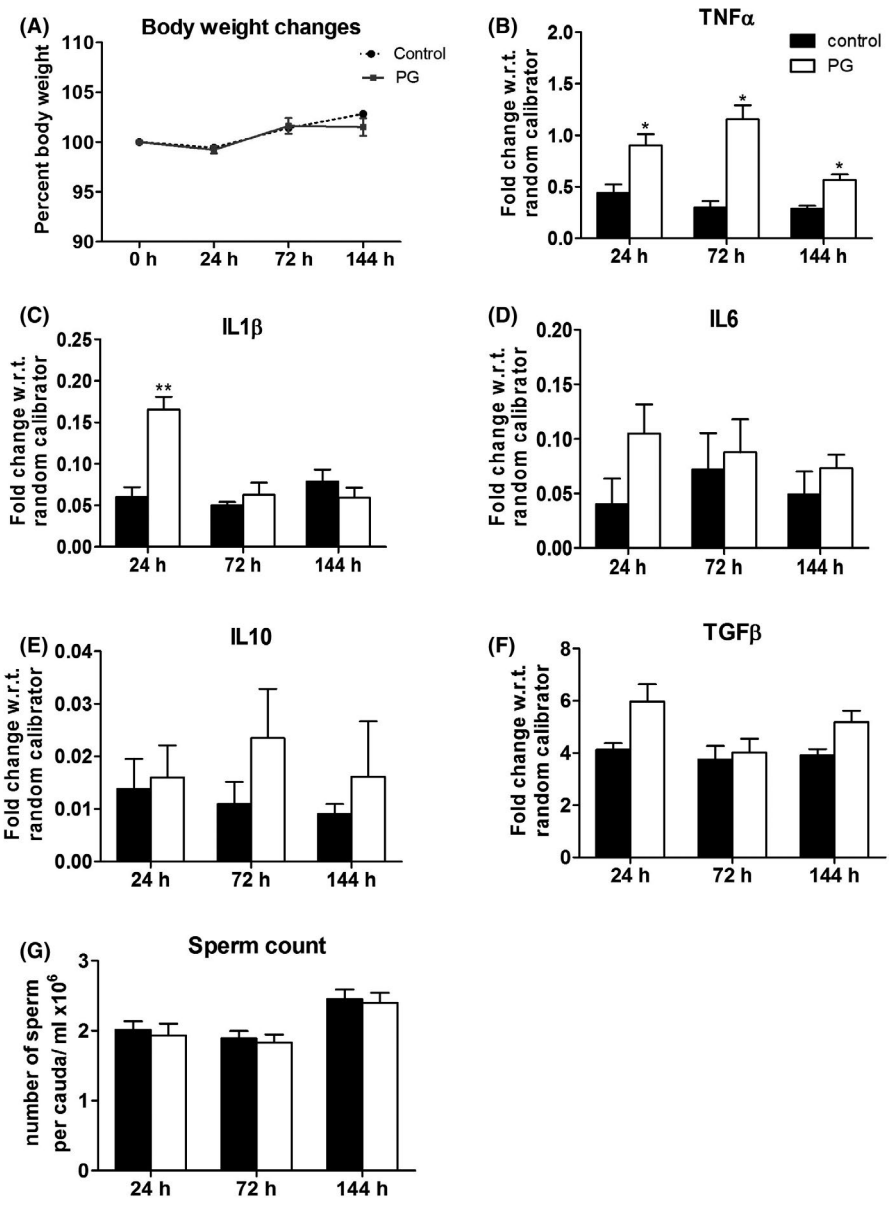

FIGURE 2 Effects of systemic peptidoglycan (PG) injection on body weight, testicular cytokine milieu and caudal sperm counts in mice. Male mice were treated with PG as described, and (A) body weight changes are shown as normalised to the weight at the beginning of the experiment (n = 5–10), and (B-F) testicular cytokine gene mRNA levels (n = 5) were assayed at various times as shown. Levels of mRNA for TNF-α (B), IL-1β (C), IL-6 (D), IL-10 (E) and TGF- β (F) were measured by qRT-PCR, and normalised values as shown were calculated. (G) Caudal sperm counts at indicated times post-PG injection (n = 5–11). Data are shown as mean ±SE. * $p < .05$, ** $p < .01$

mice. For this, 8 weeks after LPS, PG, polyIC or saline injection, each male was individually cohabited for 3 weeks with sexually mature healthy female mice of proven fertility. Pregnancies resulted from all matings set up, with either saline-treated or LPS/PG/polyIC-treated sires. The resultant litter sizes were also comparable between the various groups of sires (Figure 5A). However, the neonates from LPS-treated sires showed substantially lower birthweights than those from saline-treated sires (Figure 5B). Notably, this effect was seen with PG-treated and polyIC-treated sires as well (Figure 5B).

There is evidence that low-birthweight neonates can undergo compensatory weight gain, particularly in adipose tissue, and this phenotype is accompanied by an insulin-resistant hyperglycaemic metabolic syndrome-like phenotype.[24] We therefore followed the weight gain of these neonates over the next three weeks. It was apparent that progeny from LPS-treated sires remained smaller all through this period (Figure 5C-5E). Progeny from polyIC-treated sires tended to remain small over this period, but progeny from PG-treated sires showed comparatively higher average weight than the control group (Figure 5C-5E). Notably, there were no differences in the body weights of male versus female pups at 14 and 21 days in any of the groups (Figure S11). At 8 weeks of age, male progeny mice showed no differences in their lean weights or their lengths (Figure 6A-6B). However, female progeny mice showed marginally lower lean body weight (Figure 6D) but not in length (Figure 6E). When serum glucose levels were estimated in them, they showed no differences between the various groups of progeny (Figure 6C, F).

In order to examine the possible basis of the lower birthweight further, pregnant females were euthanised at 17.5 dpc and foetal lengths and foetal and placental weights were recorded. Placental weights were clearly lower in the groups with LPS/PG/polyIC-treated sires (Figure 7A), whereas foetal weights and lengths showed no differences between progeny of LPS/PG/polyIC-treated versus saline-treated sires (Figure 7B-C).

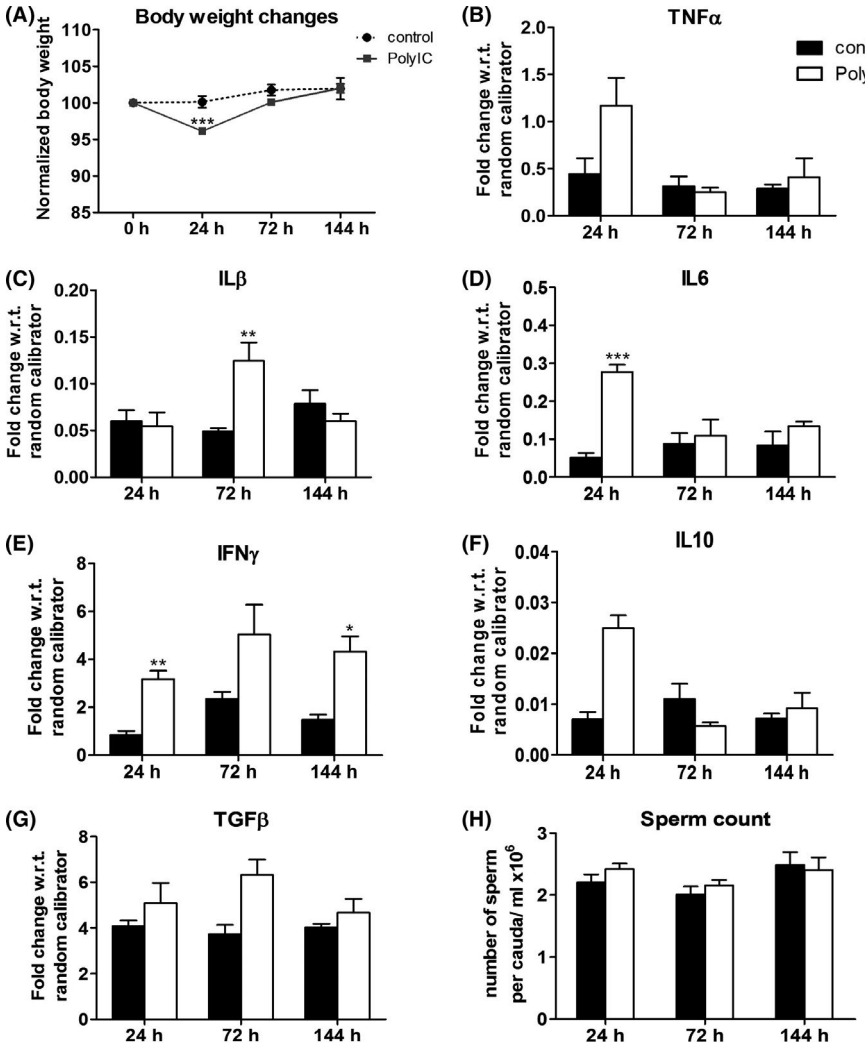

FIGURE 3 Effect of systemic polyinosinic-polycytidylic acid (polyIC) injection on body weight, testicular cytokine milieu and caudal sperm count in mice. Male mice were treated with polyIC as described, and (A) body weight changes are shown as normalised to the weight at the beginning of the experiment (n = 5–13), and (B-G) testicular cytokine gene mRNA levels (n = 5) were assayed at various times as shown. Levels of mRNA for TNF-α (B), IL-1β (C), IL-6 (D), IFN-γ (E), IL-10 (F) and TGF- β (G) were measured by qRT-PCR, and normalised values as shown were calculated. (H) Caudal sperm counts at indicated times post-polyIC injection (n = 5–11). Data are shown as mean ±SE. * $p < .05$, ** $p < .01$, *** $p < .0001$

These data suggested that the epigenetic landscape of sperm from LPS/PG/polyIC-treated mice was likely to have been altered with reference to its post-fertilisation programming for placental formation. We therefore tested the testicular levels of a major modifier of epigenetic programming that is known to respond to inflammatory stimuli, the H3 K4-specific histone methyltransferase enzyme mixed-lineage leukaemia-1 (MLL1). While MLL1 levels in the testes of LPS/PG/polyIC-treated mice did not show changes at 24 h after administration (Figure 7D), by 8 weeks after exposure, testicular MLL1 levels were substantially reduced (Figure 7E).

4 | DISCUSSION

Our data here show that there are long-term transgenerational functional consequences of even a single transient systemic inflammatory event in the testes. While the resultant local testicular inflammation is transient and subsides within a week, it leads to long-term alteration of the spermatogenetic programme. This results in a modest reduction in sperm counts and epigenetic alterations in post-fertilisation events such as placentogenesis and progeny weight.

Immune privilege in tissues is thought to be mediated by a blood-tissue barrier formed by local endothelial cell properties, ensuring that diverse blood-borne activators, cells as well as molecules, do not easily enter the privileged tissues.[25,26] One consequence of such sequestration is prevention of access of potentially autoreactive T cells to tissue-specific sequestered self-antigenic proteins.[27,28] Another consequence is the prevention of easy ingress of infectious agents and of inflammatory cells.[29] Nonetheless, there are examples of tissue-tropic infections for the testes, such as infections by *Chlamydia trachomatis* or uropathogenic *Escherichia coli*, or by viruses such as mumps virus, Coxsackie virus or Zika virus.[30-32] Similarly, there is evidence that the testes have the capacity to mediate local inflammatory responses. Testicular cells express an array of PRRs such as TLRs, RIG-I-like receptors and NOD-like receptors, which are actively involved in mediating testicular innate immune responses.[14-17,33] Further, the persistence of a number of viruses in the testes and epididymides of male mice has been shown to be associated with induction of pro-inflammatory mediators, tissue damage, diminished testosterone levels, impaired blood-testis barrier (BTB) and sperm quality.[7-10,34]

Our findings at early time points upon systemic TLR ligand administration were consistent with earlier reports,[18,20,35] by showing

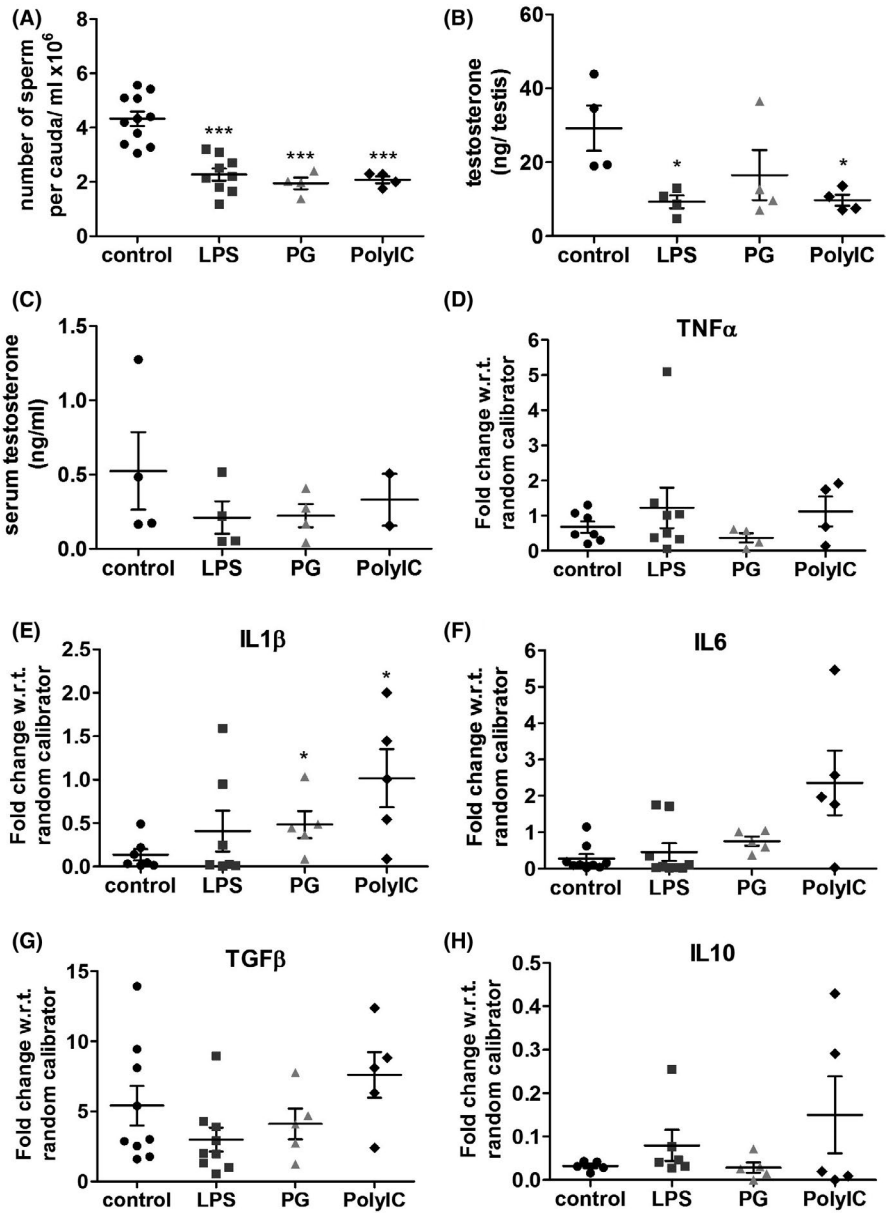

FIGURE 4 Long-term impact of a single transient systemic TLR agonist exposure on sperm counts, testosterone levels and testicular cytokine milieu in mice. Eight week after a single i.p. injection of LPS/PG/polyIC, (A) caudal sperm counts (n = 4–11), (B) testicular testosterone levels (n = 4) and (C) serum testosterone levels (n = 2–4) were measured. (D-H) Levels of mRNA for TNF-α (D), IL-1β (E), IL-6 (F), TGF-β (G) and IL-10 (H) were measured using qRT-PCR (n = 4–8). Data represent mean ±SE. *$p < .05$, ***$p < .0001$

local induction in the testes of mRNA for a number of cytokines: TNF-α, IL-1β and IL-6. Notably, TLR2 and TLR3 ligands were less potent than TLR4 activation in inducing local inflammatory responses in the testes. The next issue to examine was the extent, if any, to which this transient local inflammation modulated testicular function. Activation of TLR3 or TLR4 has been shown to suppress steroidogenesis by Leydig cells.[16] Also, sperm vitality was reduced at early time points in rats given a single dose of systemic LPS.[36] Consistent with these findings, our data showed that caudal sperm counts were significantly decreased within 24 h of systemic LPS injection, though not with PG or polyIC. However, both the local cytokine responses and the sperm count reduction came back to normal baselines within a week of induction of transient systemic inflammation. This was not only true of the testes but of the brain as well. There were local inflammatory responses that returned to baseline within a few days, and the acute functional consequences were seen only on the first day after TLR ligand administration.

Under these circumstances, there appeared no prior reason to expect any long-term testicular consequences of this transient inflammation. However, there is evidence that, five weeks after a single systemic dose of LPS, male mice can show continuing features of persistent inflammation with infiltration of immune cells in the interstitial spaces, thickening of smooth muscle layers, and dysregulated spermatogenesis with loss of germ cells in seminiferous tubules and presence of germ cells in the caudal lumen.[22] Our data did not show any persistent local testicular inflammation at 8 weeks after LPS/PG/polyIC exposure. However, we did observe a modest but consistent reduction in the caudal sperm count 8 weeks after LPS/PG/polyIC exposure, accompanied by a similarly modest but consistent reduction in testosterone levels.

Thus, our data suggest a trajectory in which there is early transient local inflammation in the testes accompanied by reduction in sperm counts, which resolves within a week, and yet leads to a reduction in sperm counts and testosterone levels 8 weeks later without

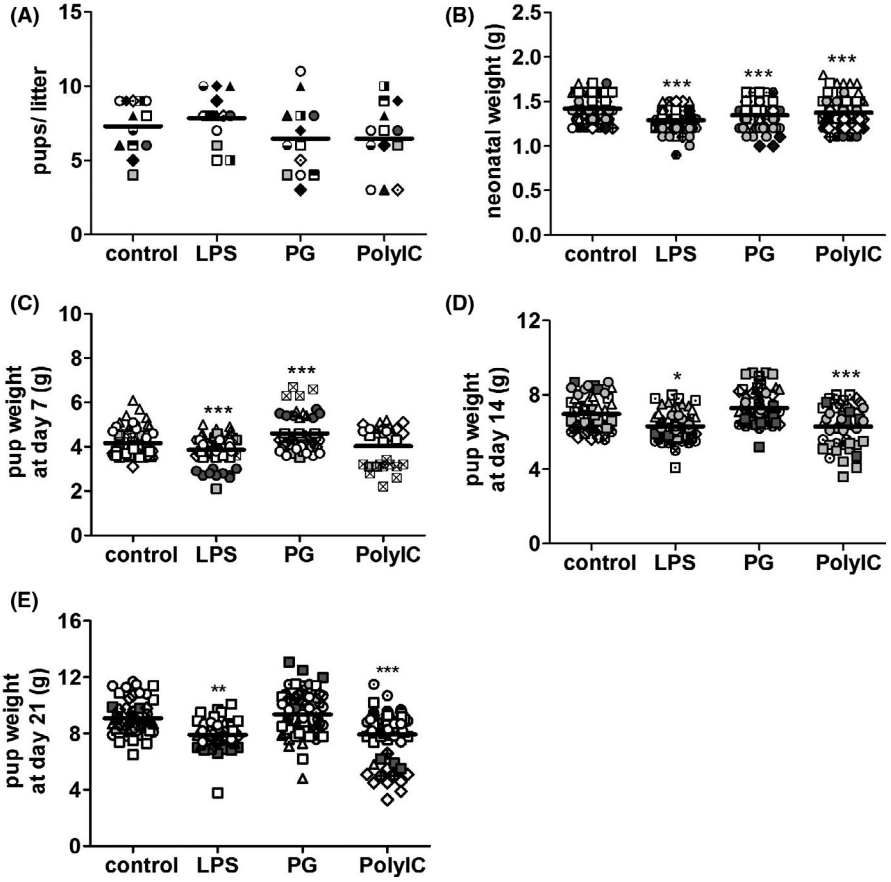

FIGURE 5 Low neonatal body weights and poor weight gain in progeny sired by mice treated with LPS/PG/polyIC eight weeks previously. Data on progeny from proven fertile females mated with male mice treated 8 weeks earlier with LPS, PG or polyIC or saline (control). A, Number of pups in each litter (n = 13 L). B, Pup weights at birth (n = 13–15 L representing 84–116 pups). C, Pup weights at postnatal day 7 (n = 6–9 L representing 29–62 pups). D, Pup weights at postnatal day 14 (n = 8–11 L representing 45–70 pups). E, Pup weights at day 21 (n = 6–12 L representing 48–70 pups). Each litter is represented by a distinct symbol. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .0001$

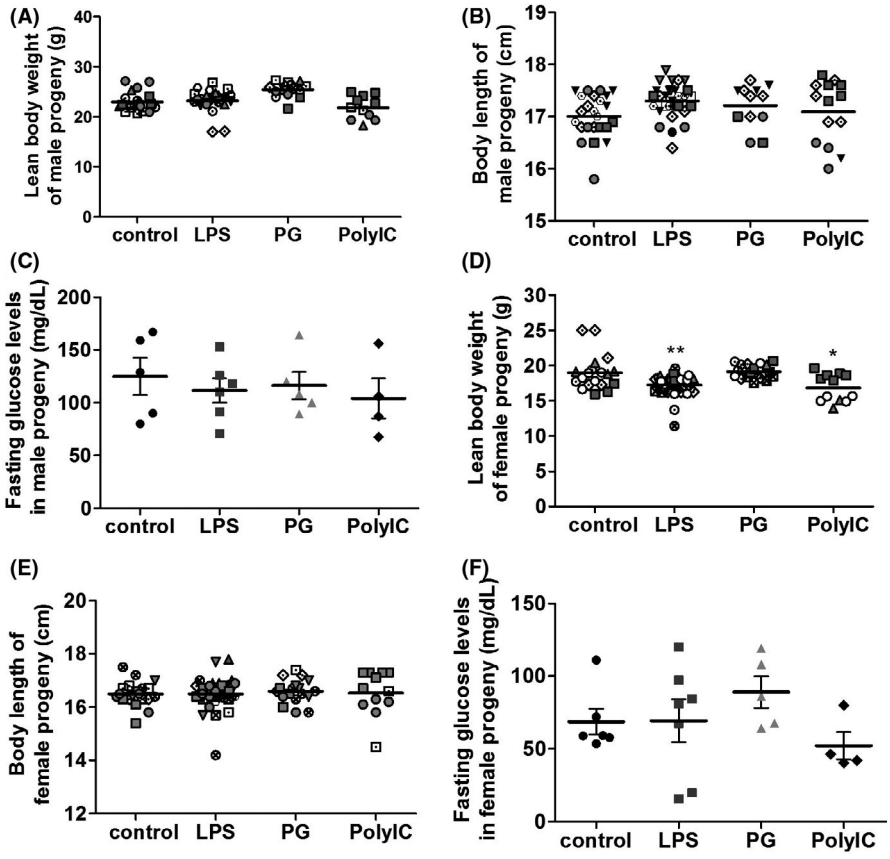

FIGURE 6 Absence of metabolic differences in adult progeny sired by mice treated with LPS/PG/polyIC eight weeks previously. Data on 8 week-old progeny from proven fertile females mated with male mice treated 8 weeks earlier with LPS, PG or polyIC or saline (control). (A) Lean body weights of male progeny. (B) Body length of male progeny. (C) Fasting blood glucose levels of male progeny after 16 h of fasting. (D) Lean body weights of female progeny. (E) Body length of female progeny. (F) Fasting blood glucose levels of female progeny after 16 h of fasting. For panels A, B, D and E, each litter is represented by a different symbol. For glucose estimation, n = 4–7. For other panels, n ≥ 12. Mean ±s.e. is shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

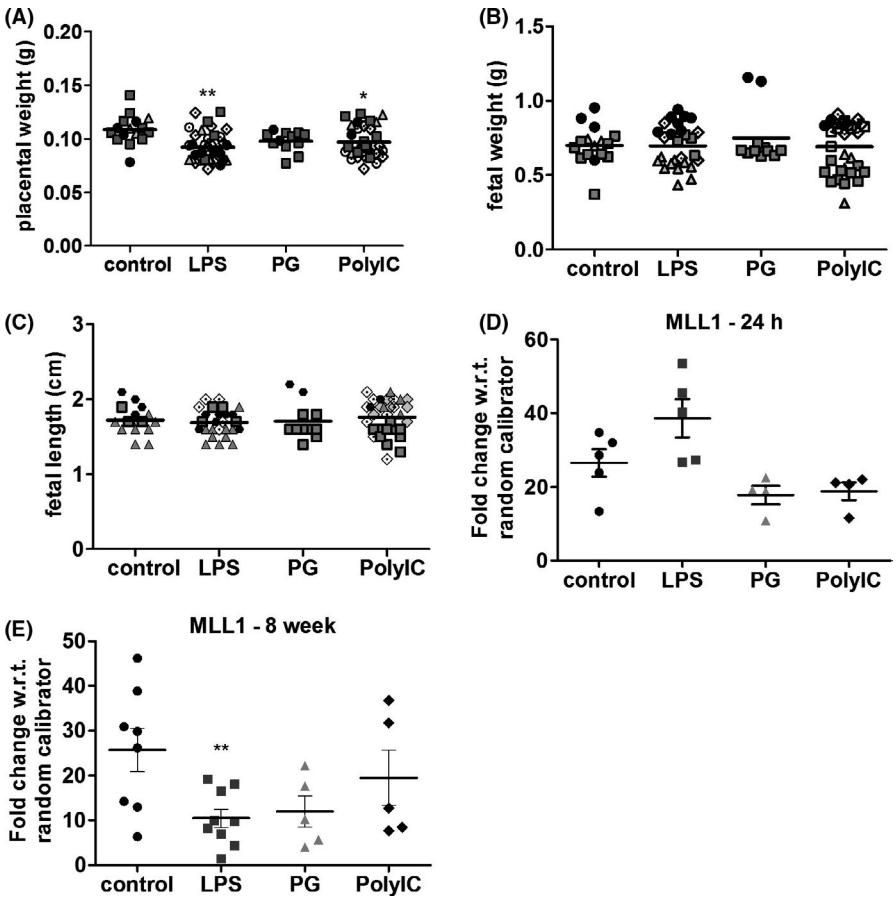

FIGURE 7 Low placental weights in pregnancies sired by mice treated with LPS/PG/polyIC 8 weeks previously, with evidence of testicular epigenetic modulation in these sires. Proven fertile females were mated with sires treated 8 weeks earlier with LPS, PG or polyIC, or saline (control). Occurrence of vaginal plug was treated as day 0.5 of pregnancy. At 17.5 days, pregnant dams were euthanised to measure (A) placental weights, (B) foetal weights and (C) foetal lengths (n = 12–33 placentae or foetuses from 2–5 dams). Data from each dam are represented by a different symbol. (D, E) Normalised transcript levels of MLL1 in the testes, 24 h (D, n = 4–5) and 8 weeks (E, n = 5–9) after treatment with LPS, PG, polyIC or saline (control). Data represent mean ±SE. $^{*}p < 0.05$, $^{**}p < 0.01$

any persistent inflammation. While the effects of TLR4 activation were the strongest impacting almost all the parameters examined, TLR2 or TLR3 activation resulted in variable effects on increases in cytokine mRNA levels, as well as on weight loss. TLR2 or TLR3 activation also did not lead to intense inflammation or sperm count reduction as TLR4 activation did. However, the later alterations in testicular function were almost equivalent in all three cases. Despite the variations observed in the acute-stage inflammatory response between different TLR ligands, the effect on the birthweight and weight gain of progeny appeared consistent. From literature, it is known that IL-6 overexpression can induce body weight reduction in obese mouse models,[37,38] while TNF-α inhibitors increase body weight and BMI in therapeutically treated humans,[39] making it difficult to implicate any one cytokine in these effects. Thus, in our study though the early inflammatory responses to polyIC were modest, the effects on progeny weights were evident nonetheless, suggesting that relatively low early inflammatory cytokine levels may be sufficient to provide signals for testicular reprogramming. The late enhancement in IL-10 mRNA levels was not consistently observed. While IL-10 mRNA levels did go up after LPS and polyIC, the increase after PG was marginal. The coupling of aggressive inflammatory factors followed by IL-10 ensures that any inflammatory response will securely be downregulated after some time.[40] However, the period of acute inflammatory responses was likely sufficient for testicular reprogramming, independent of IL-10–mediated downmodulation. Thus, very transient and modest levels of inflammatory cytokines appeared to have been sufficient to lead to durable testicular reprogramming. The precise identities and effects of the inflammatory cytokines responsible for these effects will need further elucidation using treatment of male mice *in vivo* with anti-cytokine antibodies. It should be noted that it remains formally possible that the reprogramming was not in fact mediated directly by the inflammatory cytokines but by an independent TLR-mediated pathway, especially since both Leydig cells and germ cells express TLRs.[15,16]

Persistent reduction in caudal sperm counts could plausibly be related to germ cell damage, including some apoptotic loss. Since the range of apparently normal sperm counts is wide, a twofold difference would seem unlikely to result in any detectable loss of fecundity, and in fact, mating studies did not reveal any deficits in the fecundity of TLR agonist-treated male mice. Studies with male mice carrying targeted deletions of a number of genes with important roles in testicular spermatogenesis and steroidogenesis have been reported to exhibit normal fertility even with significantly reduced sperm counts or testicular weights.[41-43] The female genital tract is known to exert strong selection pressure to promote movement of a small but high-quality fertilising subpopulation of spermatozoa to ultimately reach the fertilisation site.[44] Therefore, deficits in sperm counts or function could be masked by the proficiency of this selection system.

However, since the TLR agonist-treated male mice showed evidence of an altered programme of spermatogenesis, we further examined the pregnancies for any evidence of dysregulated

embryogenesis. Clearly, transient paternal exposure to TLR agonists led to defects in placental and foetal growth in utero. The placenta plays a key role in foetal nutrition, via active transport of nutrients and metabolic by-products across the foeto-maternal interface,[45] and changes in placental nutrient transport can directly contribute to altered foetal growth.[46] Our data suggest that transient exposure to TLR ligands led to long-term epigenetic alterations in the sperm that caused poor placental maturation.

It has become increasingly clear that sperm can transfer epigenetic information to influence foetal phenotypes,[47] and a number of examples of such modifications have been reported in both animal models and the human system.[48-51] Paternal dietary modifications have been extensively reported to alter progeny metabolic phenotypes.[50,52,53] Similarly, paternal stress has been shown to affect a number of progeny phenotypes.[54,55] There is also growing evidence that paternal epigenetic influences modify the development and maturation of the placenta and can thus lead to underweight progeny.[56,57]

Epigenetic alterations are thus potential determinants that link paternal environment to sperm quality and offspring development. The mechanisms of epigenetic alterations in sperm that affect foetal outcomes are thus of interest. The molecular pathways shown to be able to mediate epigenetic signatures in sperm include sperm RNAs, DNA methylation and histone modifications.[50,52,58] Studies with male mice fed high-fat or low-protein diets have demonstrated an increase in the levels of fragmented small tRNAs (stRNA) in sperm and altered patterns of sperm DNA methylation, associated with metabolic diseases in offspring.[52,59] When male mice maintained on restricted diet for a prolonged period of 10 weeks were mated to untreated females, they sired offspring with reduced postnatal weight and growth but increased adiposity and dyslipidaemia.[60] Importantly, another study of transient starvation model where animals were subjected to transient 24 h fasting, when mated to untreated females 2 weeks later, sired offspring exhibiting decreased serum glucose levels. However, transient fasting of sires in this study did not significantly alter the average litter size, sex ratio and body weight of the offspring.[61] Findings from these reports imply that minor body weight reduction observed transiently upon LPS or polyIC injection may not be the reason for low birthweight progenies observed in our study. This notion is further supported by the fact that the PG-challenged sires in our study though did not exhibit any body weight reduction sired progenies with low birthweight. Immune activation via polymicrobial abdominal sepsis in sires was shown to affect progeny immune phenotypes, accompanied by differentially hypo- and hyper-methylated cytosine residues spread over the sperm genome, implicating the sperm methylome as a potential carrier of epigenetic information.[62]

Our data so far provide no direct evidence of the precise epigenetic pathways altered in sperm developing eight weeks after a single transient systemic inflammatory episode. We examined the testicular expression of a potential marker that would indicate epigenetic alteration in the testes subsequent to inflammation. Mixed-lineage leukaemia-1 (MLL1), a histone methyltransferase enzyme with site specificity for H3 K4, is induced by inflammatory signals in a number of cell lineages.[63,64] It is known to be required for the epigenetic maintenance of gene activation during development,[65,66] and disruption of H3 K4 methylation in sperm has been reported to have transgenerational consequences.[58] Indeed, our findings of decreased levels of MLL1 in the testes of TLR agonist-injected mice 8 weeks after exposure show that major epigenetic modification of sperm programming is likely to have taken place. The precise pathways involved thus become future directions of major interest.

Underweight human neonates that gain weight postnatally in compensatory fashion have been shown to acquire a more adipose phenotype, and to be more prone to early acquisition of insulin resistance.[67,68] We therefore followed the postnatal weights of the underweight progeny mice almost to the end of the weaning period and tested their lean weights, lengths, and blood glucose levels at early adulthood. The underweight progeny of LPS- or polyIC-treated sires remained underweight, while those of PG-treated sires tended to recover weight. However, during later periods, the lean weights of progenies were similar, suggesting that there was no increase in adiposity, and blood glucose levels were comparable, indicating that, in this model of intrauterine growth retardation, postnatal metabolic syndrome was not a prominent feature.

The data reported here have significant relevance to two commonly observed human situations. Men with a history of infections and with pathological semen profiles are frequently subjected to assisted reproductive technology (ART) procedures, and ART-conceived children are reported to have lower birthweights and higher peripheral fat, blood pressure and fasting glucose concentrations than naturally conceived children.[69] Thus, it is possible that inflammation-modified sperm epigenetic programming in such situations may contribute to post-ART low birthweight outcomes. If so, sperm epigenetic assessment may be of help in these ART procedures.

A second issue is related to the higher proportions of low birthweight (LBW) and small-for-gestational-age (SGA) newborns seen in some parts of the world. Proportions of newborns with LBW or SGA are high in many developing countries including India.[70] Lower standards of hygiene and higher frequency of infections in individuals from lower socio-economic conditions may contribute to frequent inflammatory episodes involving testis in apparently normal healthy men. The resultant testicular reprogramming may be a contributory factor in the higher frequencies of low birthweight newborns in such conditions. Of course, our data only address the effect of a single transient exposure to TLR agonists on durable testicular reprogramming, and the consequences of repeated and/or chronic infections remain unexplored as yet. Repeated exposure to LPS in experimental situations has been shown to lead to endotoxin tolerance.[71] However, it is unlikely that the epigenetic spermatozoal modifications resulting from a single transient acute inflammatory event as documented here would show reversal due to LPS tolerance. However, this needs to be explored in future work.

In conclusion, our findings demonstrate that exposure to transient systemic inflammation has persistent sperm-mediated consequences

on progeny health. Maternal health, especially in low-income communities with high levels of exposure to infectious diseases, is widely linked to neonatal health, especially to intrauterine growth retardation.[72] LBW in infants has been generally considered to be the result of maternal and consequently foetal under-nutrition. However, attempts to improve these neonatal weight outcomes by improved maternal health and nutrition are only partly successful.[73-75] Our data indicate that paternal high-frequency pathogen exposure may well be a factor contributing epigenetically to the developing country problem of frequent intrauterine growth retardation.

ACKNOWLEDGEMENTS

Financial support from the DBT-RA Program in Biotechnology and Life Sciences Department of Biotechnology, Government of India to SuR and Cognitive Science Research Initiative, Department of Science and Technology, Government of India to AG and NS (Grant No. SR/CSRI/331/2016(G)) is gratefully acknowledged. Indian Institute of Science Education and Research, Pune, is funded by Ministry of Education, Government of India.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SuR did most of the experimental work, analysed data and wrote initial draft of the manuscript. MU and DSB contributed specific methodologies and some critical experiments. NS, CSY and AG provided conceptual and technical input in experimental design. SR and VB conceived the research question, planned experiments, analysed data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are included in this published article and its supplementary information files. The corresponding author can provide raw data to researchers on their justified request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Rokade S, Upadhya M, Bhat DS, et al. Transient systemic inflammation in adult male mice results in underweight progeny. *Am J Reprod Immunol*. 2021;86:e13401. https://doi.org/10.1111/aji.13401