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RESEARCH ARTICLE

Responses of Chicken Sertoli Cells and Fibroblasts after Transfection with Plasmids pEGFP-N3-HNP-1

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ABSTRACT

Chicken Sertoli cells (SCs) and fibroblast cells (FCs) were transfected with two different plasmid vectors to study their comparative responses to transfection and to heterogenous protein appeared *in vitro* cultures of both cell lines. Sertoli cells and FCs (control) were transfected with plasmids pEGFP-N3-HNP-1 and pEGFP-N3 and efficacy was recorded. Subcellular localization of both proteins was observed. IL-1β, IL-1RN, Fas, FasLG (FasL) and Caspase-3 expressions were examined using Real-Time PCR. The fibroblast cells were more efficient in transfection activity than SCs. Moreover, plasmid pEGFP-N3 had higher capability of transfection compared to pEGFP-N3-HNP-1 plasmid. The cells confined the poisoning protein in large particles and non-poisonous protein appeared all over cell in thin particles. The inflammatory response of SCs to non-poisonous heterogenous protein was lower than to poisonous heterogenous proteins compared to FCs. The FasL response of SCs to poisonous protein was faster than to non-poisonous proteins. It is concluded that Sertoli cells may create strong resistance against transfection than fibroblast cell, while the former contain large amounts of harmful/poisonous proteins that may modulate a quick inflammatory response. The quick inflammatory response may lead to apoptosis in Sertoli cells which is thought to be a way to get rid of unhealthy cells.

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INTRODUCTION

Sertoli cells are the main structural and biochemical component involved in the development of testes and spermatogenesis (Kopera *et al*., 2010; Alves *et al*., 2013), a unique function taking place in microenvironment behind the blood-testis barrier (BTB). A variety of cytokines like interleukin (IL)-1α, IL-1β and IL-1 antagonistic molecule (IL-1Rα) may facilitate germ cell movement across the seminiferous epithelium during cellular events such as germ cell differentiation, which may participate in the disassembly and assembly of the BTB by controlling the production of IL-1 by Sertoli cells. In the testis, these ILs (-1α, -1β and -1Rα) remain balanced to a required level, but any infection or inflammation may disturb the cellular homeostasis by a rise in ILs concentration (Söder *et al*., 2000). Therefore, the perturbation of IL-1 in Sertoli cells negatively affects the integrity of BTB and results in the sloughing of germ cells (Lie *et al*., 2011). The phenomenon how the Sertoli cells cope with the intrinsic stresses such as when the blood testes-barrier is damaged is not fully known yet.

Human neutrophil peptide-1 (HNP-1) is a tiny anti-microbial peptide with little cytotoxicity to induce a certain amount of stress in different cell lines. In view of this, we placed the chicken Sertoli cells under transfection stress with pEGFP-N3-HNP-1 (VpN3H1) (Tanaka *et al*., 2010; Lu and de Leeuw, 2013) and the empty vector plasmid pEGFP-N3 (VpN3) to study the response of Sertoli cells against transfection by comparing with the fibroblast cells chicken as a control. Cytokines and apoptosis arrays (IL-1β, IL-1RN, Fas, FasL and Caspase-3) were also studied along with heterogeneous cellular proteins.

MATERIALS AND METHODS

Experiments performed at Animal Sciences Laboratory were approved by college of Animal Science, Northeast Agriculture University, Harbin, China.

Preparation and purity of SCs and FCs: The testes of 6 weeks-old, immature Arbor Acre (AA) chicken were collected to prepare Sertoli cells following a previous report (Guibert *et al.,* 2011) with slight modifications. The cells were cultured in Dulbecco's Modified Eagle Medium (F12/DMEM) supplemented with antibiotics and 10% fetal bovine serum maintained at 37°C in a humidified atmosphere with 5% CO_2. Following Bai *et al*. (2011), the embryos from 8-10 days incubated eggs in stage 26 were collected and fibroblast cells were prepared according to the American Type Culture Collection procedure (http://www.rktech.hu/dokumentaciok/LGC/ATCC_Cell_Culture_Technical_Resource_catalogue.pdf), with the purity of SCs and FCs was over 90%. The illustrations for SCs and FCs preparations and morphology corresponded to healthy cells under phase contrast microscope are given in Fig. 1.

VpN3H1 and VpN3 transfection and experimental procedure with Sertoli and fibroblast cells: The SCs (study group) and FCs (control group) preparation were transfected with two plasmids viz. VpN3H1 (Yu *et al.,* 2014) and VpN3, using the lipofectamine (lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) (Tsuchiya *et al.,* 2002). Following transfection Dulbecco's Modified Eagle Medium (DMEM) was changed 4-6 h subsequently for 48 h and observations were made every 24 h. The green fluorescent protein (GFP) expression of VpN3H1 and VpN3 transfected cells was observed under an inverted fluorescence microscope equipped with a digital camera (EVOS) with excitation wave length of 470-531 nm.

The morphology of positive cells was observed in both cell groups and the images were taken from 10 visual fields to calculate the transfection efficiencies taken at 24, 48 and 72 h, which were calculated as the ratio between the number of positive cells and the total number of cells: E=P/T×100% (E: Transfection efficiency; P: total for positive cell; T: total cell number; Wu *et al*., 2008). The experiment was repeated 5 times for the confirmation of results and data was averaged. After 72h post transfection, G418 antibiotic was added to the medium and transfected cells were selected for further observations like cell status and cell divisions.

Localization of VpN3H1 and VpN3 protein: In cell culture, certain cluster of cells often formed, which were fixed in ice-cold methanol for 10 min. and washed thrice with PBS containing 0.1% TritonX-100. Nuclear staining was done with 4', 6-diamidino-2-phenylindole (DAPI) at 37°C in the dark and cells were observed by laser scanning confocal microscopy after 10 min (Huang *et al*., 2013).

Plasmid transfection induced expression of inflammatory cytokines viz. interleukins (IL)-1β, IL-1RN, FasL, Fas and Caspase-3 genes: Total RNA was extracted by using Trizol reagent from SCs and FCs after transfection with VpN3H1 and VpN3 at 48 h and 96 h. RT-PCR (Ou *et al*., 2011) and PCR was performed to prepare cDNA which was subsequently used in PCR reactions for cytokines and apoptotic proteins arrays. The PCR products were electrophoresed on 1.5% agarose gels. Amplification was measured using real-time quantitative reverse transcription PCR (q-PCR) (ABI PRISM 7500 real-time PCR system Applied Biosystems, CA, USA; TaKaRa Kit) and was used to detect the expression of the IL-1β and IL-1RN genes and apoptotic factors Fas, FasL and Caspase-3. The Primer Premier Software (Oligo, China) was used to design specific primers and β-actin based on known sequences (Table 1). The reactions were performed in triplicate for each sample.

Statistical analysis: Analysis of variance (ANOVA) was applied by using CoStat package on the data and expressed as the mean±SD. For RT-qPCR analyses, the difference between threshold cycles ($^{\Delta}$Ct) was calculated by subtracting the Ct value of target gene from that of the reference gene, and subjected to ANOVA to generate $^{2\text{-}\Delta\Delta}$Ct values (Livak and Schmittgen, 2001).

RESULTS

VpN3H1 and VpN3 plasmid transfection efficiency: At 24, 48 and 72 h after transfection with VpN3H1 and VpN3, the green fluorescence protein (GFP) expression in both group of cells (SCs and FCs) is shown in Fig. 2A. At 24h post transfection, GFP start appearing within cells of both types which turned to dense fluorescence at 48h post transfection. After total RNA was extracted from the SCs and FCs, RT-PCR and PCR showed the presence of pEGFP-N3-HNP-1, 294 bp was expressed in both kinds of cells and was not expressible in the blank cell group. β-actin expression was positive (Fig. 2B) and could be used to check the efficacy of an RT-PCR reaction. The transfection efficiency of FCs with both VpN3H1 and VpN3 plasmids was higher than for SCs (P<0.05) at 24, 48 and 72-h (Fig. 2C). At 48h post transfection, the efficiency of SCs and FCs towards VpN3H1 plasmid transfection was significantly lower than with VpN3 (5.93±0.25, 25.10±0.81; 9.03±0.17, 30.60±0.34, respectively*;* P<0.05).

Detection and localization of VpN3H1 and VpN3 proteins after transfection: The localization of the proteins expressed via VpN3H1 and VpN3 plasmids in fixed SCs and FCs were observed under laser scanning confocal microscope, as indicated in Fig. 3A-3F. Both cell types transfected with VpN3H1 showed fluorescence lumps appeared initially in the cytoplasm in close vicinity to nuclear membrane, which later merged with nuclear membrane to get entry into the nucleus and finally karyoplasm formation out of the nucleus (Fig. 3A-3D). In VpN3 transfected cells, the fluorescent particles dispersed all over the cytoplasm and nucleus (Fig. 3E and 3F), but they were much smaller compared to VpN3H1 transfected cells (Fig. 3B and 3C). All fluorescent cells transfected with VpN3H1 plasmid died within two weeks after selection with G418 (Fig. 3G). The clones were achieved from the VpN3 plasmid transfected surviving cells.

Cytokines and Apoptotic Array Analyses at 48 and 96 h Post Transfection: Cytokines like IL-1β and IL-1RN were arrayed after 48 and 96h post transfection, the ANOVA results are shown in Fig. 4. At 48h post transfection, compared

Table 1: List of gene specific primers

Gene*	Sequences(5'-3') of primer	Accession number	Length(bp)
β-actin	CCGAGAGAGAAATTGTGCGTGAC TCGGGGCACCTGAACCTCTC	L08165	166
HNP-1	GGAATTCTATGAGGACCCTCGCCATCCTTGCTGC CGGGATCCGCAGCAGAATGCCCAGAGTCTTC	NM_004084.3	294
IL-1β	GGCACTGGGCATCAAGGGCT AGGGAGGTGCAGATGAA	NM_204524.1	210
IL-1RN	GCCTCCGCGCCGTTCACCT GGAGGTGCAGAGGAA	HE608245	93
FasL	AGGAAGCAAGGAAGGCAGCA GGAAGAGCACATTGGAGTA	NM_001031559.1	171
Fas	TTCCCACACACACTGCACATAA CACACCGAGAAGAATTGCAGTAA	NM_001199487	342
Caspase-3	CCACGCTCAGGGGAAGATGTAT CGGTATCTCGGTGGAAGTTCTTA	NM_204725	178

*Defensin, alpha 1 (HNP-1), interleukin 1, beta (IL-1β), interleukin 1 receptor antagonist (IL-1RN), Fas ligand (TNF superfamily, member 6) (FasLG), Fas cell surface death receptor (Fas), caspase 3, apoptosis-related cysteine peptidase (Caspase-3).

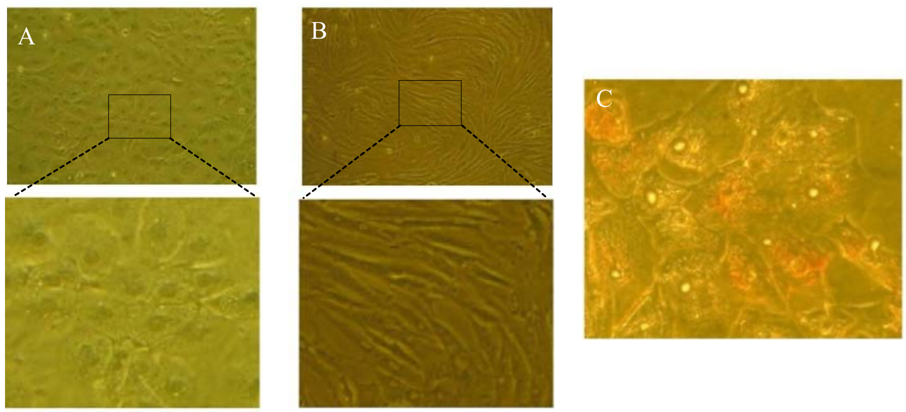

Fig. 1: Cell Morphology. (A) Sertoli cells. (B) Fibroblast cells. (C) Sertoli cells showing positive Oil Red O staining. (×100), (×10) μm.

to FCs (P<0.01) higher expression of IL-1β was observed in SCs transfected with VpN3H1, but the expression of IL-1β in FCs transfected with VpN3 was higher than in SCs (P<0.01). At 96h post transfection with VpN3H1 and VpN3, the expression of IL-1β in SCs was higher than in FCs (P<0.01). At 48h post transfection, expression of IL-1RN in FCs transfected with VpN3H1 or VpN3 was higher than those of SCs (P<0.01), but the expression of IL-1RN in SCs was higher than in FCs at 96h (P<0.01).

The ANOVA results of apoptotic array for Fas, FasL and Caspase3 expression at 48 and 96h post transfection are shown in Fig. 4. At 48 and 96h post transfection with VpN3H1 and VpN3, a little Fasl expression was detected in FCs due to their high (90%) purity. FasL expression was higher in SCs at 48 and 96h post transfection with VpN3H1 but lower expression with VpN3 at both time points (Fig. 4 FasL). At 48h post transfection with VpN3H1, Fas expression was higher in SCs than in FCs (P<0.01) and there was no difference in Caspase-3 expression between the two types of cells. Both cell types showed similar expression (P>0.05) of Fas and Caspase-3 after 48 h post transfection with VpN3 plasmid. Transfection with VpN3H1 after 96 h, Fas and Caspase-3 expression was higher in SCs than in FCs (P<0.01). On the contrary, FasL expression was higher in SCs but Fas and Caspase-3 remained the same (P>0.05) in both cell types transfected with VpN3 plasmid.

DISCUSSION

The transfection efficiencies of FCs with pN3H1 and pN3 plasmid were both higher than in SCs (Fig. 2C), which was thought to be due to the difference in permeability and composition of cellular membranes, which may be a special physiological function of SCs or the BTB. The cytoskeleton of SCs has structural and functional similarity to most of the other cells along some unique characteristics e.g., presence of tight and gap junctions, desmosome-like junctions and certain ectoplasm projections that may form large junction complexes. The BTB forming tight junctions aid in spermatogenesis, which separates the seminiferous epithelium into two compartments: basal, containing spermatogonia, preleptotene spermatocytes, and adluminal, containing mitotic and postmitotic germ cells (Mruk and Cheng, 2010). Previously, *in vitro* studies reported the presence of such junctions in rats and human cells (Miwa *et al*., 1998), but further in-depth research is needed whether similar tight junctions can be formed in *in vitro* culture of SCs. Our results also showed that transfection efficiency of both cell types against VpN3H1 plasmid was significantly lower than with VpN3 plasmid at 48h post transfection, which is quite possibly due to larger size of the VpN3H1 vector compared to VpN3. Cytoplasm and nucleoplasm of both cell types contain the dispersed tiny particles of pN3 proteins and the clones were obtained one month later after selection with G418 from each type of cells, hence pN3 did not disturb the cell physiology and biochemistry. Part of the HNP-1 protein is a kind of human defensin peptide from neutrophil cells with or without low cellular toxicity in animal cells (Grigat *et al*., 2007; Tanaka *et al*., 2010). The presence of HNP-1 proteins in the form of particle blocks within the cytoplasm may be due to the protecting function of SCs

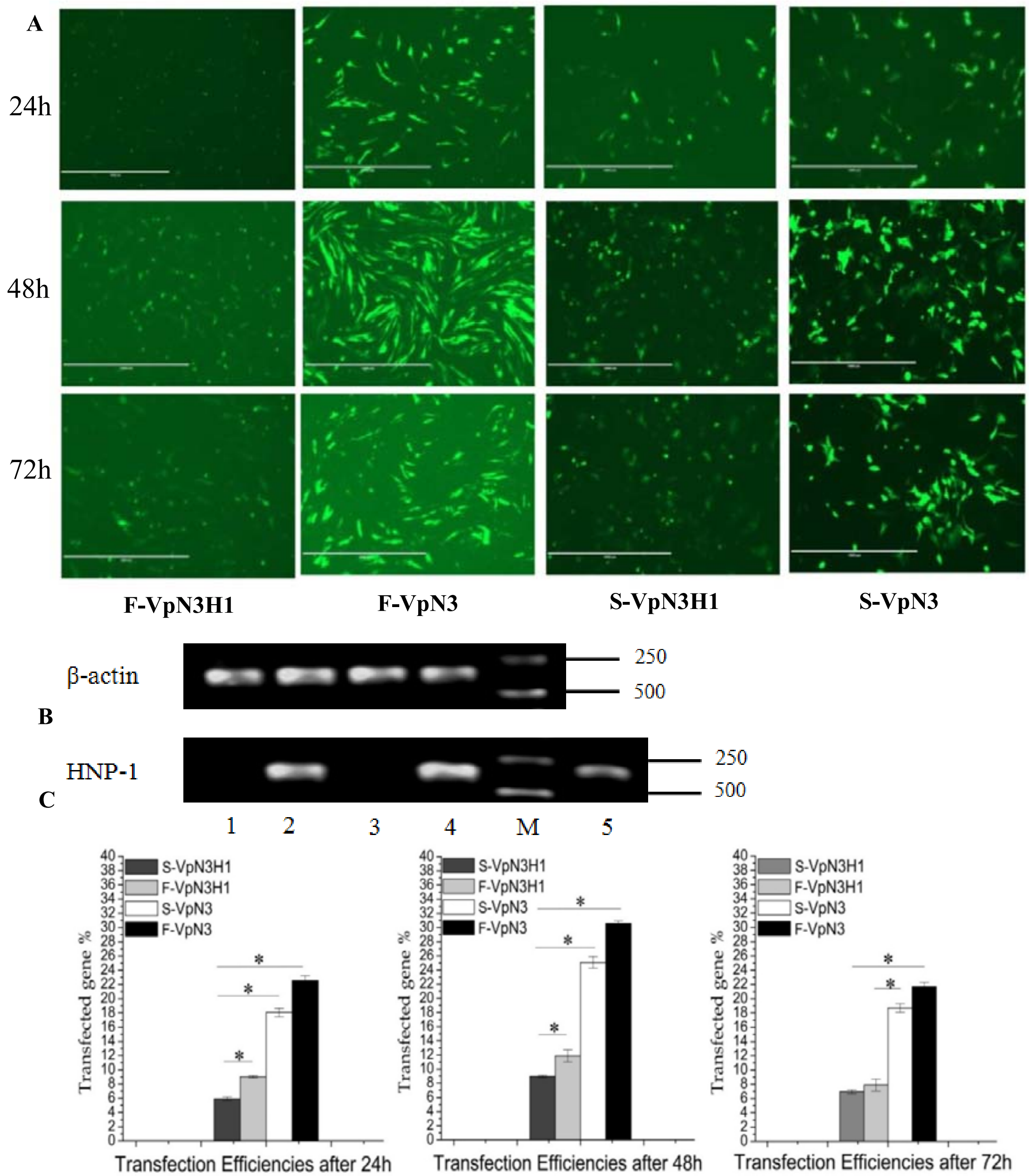

Fig. 2: Expression of Transfection efficiency. (A) Observation of the cell fluorescence expression at 24, 48 and 72h. (B) PCR analysis of positive clones; 1- Blank fibroblast cells. 2- Fibroblast pEGFP-N3-HNP-1. 3- Blank Sertoli cells. 4- Sertoli cells pEGFP-N3-HNP-1. 5- HNP-1 DNA. M-D2000 Marker. (C) Transfected SCs and FCs with the VpN3H1 and VpN3 plasmids after 24, 48 and 72h. (S = SCs, F = FCs). Values represent mean±SE (n = 4). $^*P<0.05$ compared with the fibroblast cells as the control group and empty vector group N3.

which gather certain poisonous proteins together. It has been shown that SCs contain different types of lysosomes and digest the material in the cytoplasm through active phagocytosis (Fan *et al.,* 2011).

The presence of the pN3H1 protein in the cytoplasm and around the nucleus has no effect on nuclear division, rather it has damaging effects within nucleus (Fig. 3), as nuclear entry of proteins may rupture the nuclear membrane, causing the nucleoplasm to scatter and mix up with cytosolic contents. VpN3 transfection was more successful in SCs and FCs compared to VpN3H1, even though a previous study has reported successful transfection of human defensin in other animal cells (Ma *et al.,* 2010). We proposed that SCs could be targeted for genetic modification because both kinds of transfected cells gave the clones one month later with G418 selection. Sertoli cells have immune exemption and can engulf some proteins such as dead spermatogenic cell components or residual cytoplasmic proteins, giving the explanation for the enhanced IL-1β response in FCs compared to SCs 48 h post transfection with VpN3. The converse was observed with poisonous proteins; SCs responded to VpN3H1 protein more quickly and strongly than FCs so that enhanced IL-1β appeared in the former cell line. IL-1β is an important mediator of the inflammatory response and both IL-1α and -1β are produced by testicular SCs to form a local network of cytokine immune function in testis (Zhang *et al.,* 2014). After specific time, the inflammatory response (IL-1β) of

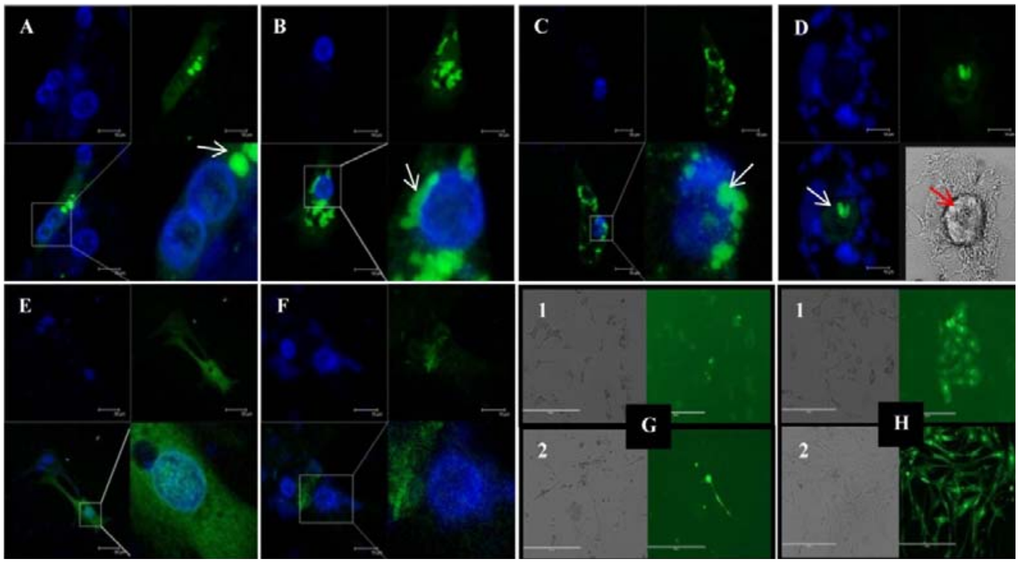

Fig. 3: Subcellular localization of the VpN3H1 and VpN3 plasmids after transfection. The cell nucleus counter stained with DAPI (blue). White arrows indicate VpN3H1 or VpN3 protein location (green). Red arrows indicate the nucleus. (A) The fluorescence lumps appear in cytoplasm or near nucleus membrane. (B) Fluorescence lumps touch nucleus membrane. (C) Fluorescence lumps enter nucleus. (D) Fluorescence lumps in nucleus make the chromatin out. (E) The thin fluorescent particles with VpN3 were dispersed all over the cytoplasm and nucleus of SCs. (F) The thin fluorescent particles with VpN3 dispersed all over the cytoplasm and nucleus of FCs (S = SCs, F = FCs). (G) All fluorescent cells with VpN3H1 transfection died in two wk (1: SCs, 2: FCs). (H) The cell with VpN3 transfection survival and formed clones one month after G418 selection (1: SCs, 2: FCs). Bar=×100 in A, B, C, D, E and F. Bar=×20 in G and H.

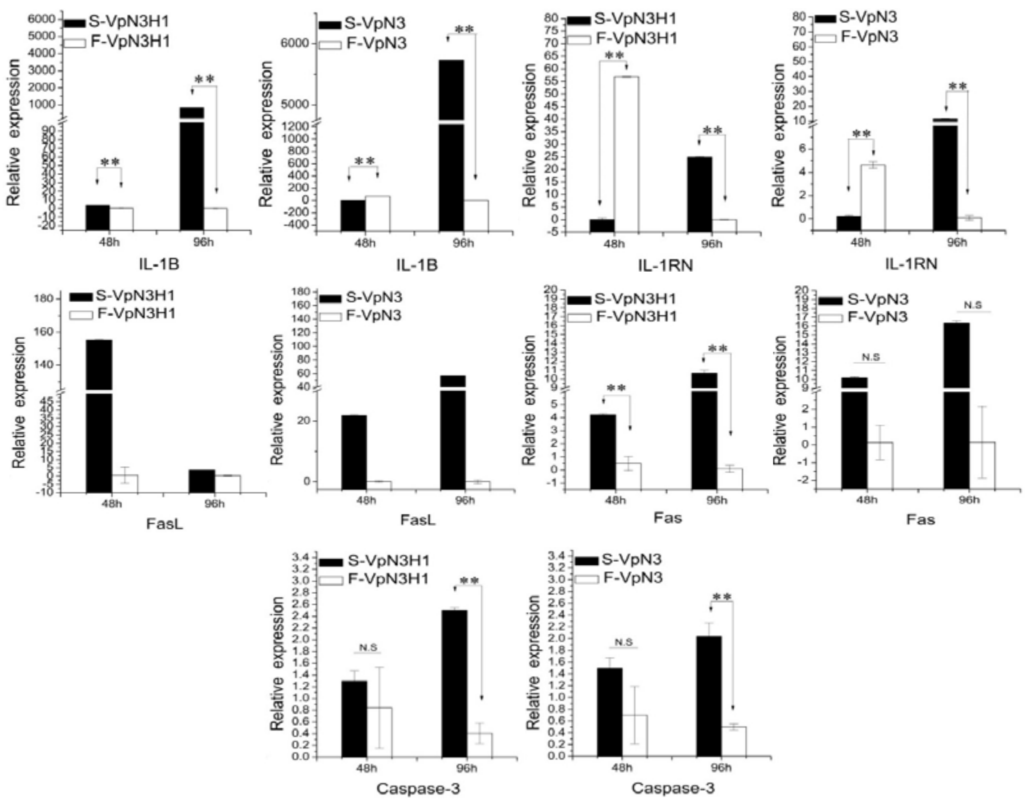

Fig. 4: Relative mRNA levels (gene of interest/β-actin) of expression cytokine and apoptosis factors in the Sertoli cells and fibroblast cells after transfection with Plasmids HNP1 and empty vector group N3. Values represent mean±SE (n=3). **P< 0.01 compared with the fibroblast cells as control group and empty vector group N3.

SCs to heterogenous proteins may increase as our results illustrated during 96h post transfection analyses. The IL-1RN responses to both kinds of transfection plasmid proteins were slower in SCs compared with FCs as seen at the 48h time point but as the time proceeded to 96h, the response was more strong. This is because the SCs have different cellular physiology and responses than FCs. The IL-1β and IL-1RN results elaborated that the inflammatory response of SCs to the non-poisonous heterogenous protein was slow, whereas their response to poisonous heterogenous protein was quick when compared to FCs. The inflammatory response of SCs to both kinds of proteins became stronger than that of FCs with increasing time.

At the 48h time point, the poisonous heterogenous proteins strongly stimulated SCs for expression of FasL for protection which decreased markedly at 96h post transfection. But the FasL response of SCs to non-poisonous heterologous protein increased gradually rather than a spontaneous response as with poisonous heterologous proteins (Fig. 4, FasL). Sertoli cells showed higher expression of Fas and Caspase-3 than FCs at 48h to 96h post transfection (Fig. 4, Fas, Fig. 4, Caspase-3). This indicates that SCs showed a stronger apoptotic tendency than FCs through the FasL/Fas-Caspase-3 pathway *in vitro*, giving an idea to get rid of unhealthy SCs by this apoptotic pathway. It can be speculated that the FasL/ Fas/Caspase-3 pathway was engaged when heterogenous proteins appeared in SCs. FasL-induced apoptosis of Fas-bearing lymphocytes is an important mechanism for the suppression of immune responses. FasL is abundantly expressed in the testes; this system was demonstrated to be critical for maintaining testicular immune privilege by inducing lymphocyte apoptosis via FasL expression (Zhao *et al.,* 2014). This shows that the protein HNP-1 stimulated pathway of apoptotic factors through FasL, which ultimately stimulated the production of IL-1β in SCs (Cheng and Mruk, 2012).

We hypothesize that FasL induces apoptosis in other types of Fas-bearing cells such as dying spermatogenic cells or SCs. From our experiments, we demonstrated that more Fas was produced in SCs than in FCs at 48 and 96h post transfection, which means that SCs have a very strong ability for self-apoptosis through the modulation of Fas expression in case of excessive stresses or damages. The renewal of SCs *in vivo* after sexual maturity is not certain. If they indeed undergo renewal, maybe that unhealthy SCs can be cleared via activation of the FasL/ Fas/Caspase-3 pathway. FasL is expressed spontaneously upon heterogenous proteins appearance in SCs to safeguard cells, and it seems that unhealthy SCs are eliminated through enhanced Fas expression.

Conclusion: In conclusion, SCs had a strong resistance against plasmid transfection than FCs, but former could be targeted for transgenic of some genes. VpN3H1 plasmid showed more transfection response from both cell lines compared to VpN3 plasmid. In vitro, unlike FCs, SCs showed higher self-protection along with higher apoptosis rates through the FasL/Fas/ Caspase-3 pathway which additionally promotes the production of IL-1β.This indicated that the FasL/Fas/ Caspase-3 pathway was responsible for depriving unhealthy SCs in vivo. This would present an avenue for the significance of the FasL/ Fas/ Caspase-3/ IL-1β and IL-1RN pathway activity we report in this study. Therefore, further research is needed to establish if the SCs undergo renewal.

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Author's contribution: AK, ZG and YN developed the conception and design of the study, analyzed data and co-drafting of the manuscript. AK, ZP and ZJ participated in collection of the experimental samples while ZX and AK contributed reagents/materials/analysis tools. AK and NK wrote the manuscript. All authors revised the manuscript and approved it.

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