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The Effect of Pulsatile Hormonal Stimulation on Gene Expression in Cultured Rat Sertoli Cells

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Abstract

FSH and Testosterone (T) are the major endocrine regulators of spermatogenesis. Both these hormones act via testicular Sertoli cells (Sc) to regulate the division and differentiation of male germ cells (Gc). A number of FSH- and/or T-responsive genes, expressed specifically by Sc having critical role in regulating sperm production, have been identified. We have previously reported the efficacy of a novel *in vitro* administration of pulsatile hormonal treatment protocol (exposure of FSH and T in combination for 30 minutes/3 hr upto 24 hr) on gene expression by cultured rat Sc as compared to that with the conventional constant (for 24 hr) hormonal exposure. In the present study, we further demonstrate that such pulsatile stimulation of hormones to cultured rat Sc show more prominent impact in terms of augmenting the expressions of Sc-specific genes critical for spermatogenesis, like *Inhibin-β-B*, *Androgen binding protein* (*Abp*) and *Stem cell factor* (*Scf*) at the 11th hr (hr) of total treatment duration i.e., 24 hr. This report thereby pinpoints the specific treatment regime for evaluating the maximal hormone-induced gene expression in cultured Sc systems. This new protocol of hormonal stimulation (for 11 hr only instead of 24 hr) will significantly improve our current practice of using primary Sc culture systems for detecting hormonal responsiveness or signaling with respect to gene expression *in vitro*.

Keywords: FSH, Gene Expression, Sertoli Cells, Spermatogenesis, Testosterone

1. Introduction

Testicular Sertoli cells (Sc) are the sole target of both FSH and testosterone (T)[1]. The synergistic action of FSH and T induces the secretion of various growth factors and metabolites by Sc to promote the initiation and maintenance of male germ cell (Gc) division and differentiation[2]. FSH acts via FSH receptor (*Fshr*), which is a G protein-coupled receptor and stimulates Adenylyl cyclase-mediated production of cAMP. This second messenger further activates a sequence of signaling events that up-regulates genes like *Inhibin β-B, Androgen binding protein (Abp), Stem cell factor (Scf)*, etc., to regulate Gc proliferation, differentiation and survival either directly or indirectly[2]. On the other hand, T acts via Androgen receptor (*Ar),* which is a steroid-inducible transcription factor that translocates to Sc nucleus upon T binding and subsequently binds to specific DNA sequences to initiate androgen-dependent transcriptional events[2].

During the last decade, a lot of Sc-specific genes regulating the spermatogenic output and, thereby, male fertility have been identified by differential transcriptome

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analyses generated from various knockout mice models ablating the action of either FSH or T[3]. However, hormone induced *in vitro* expression profile of most of these genes were found to be inconsistent when assessed at different time points with constant exposure of hormones (FSH and T, either alone or in combination) in primary Sc cultures[2,7]. We have recently demonstrated the advantages of a novel pulsatile stimulation of hormones (FSH and T in combination for 30 minutes of hormonal exposure/3 hr up to 24 hr) in cultured rat Sc in terms of evaluating hormone-mediated gene expression over traditional 24 hour of constant supplementations[8]. Herein we report further that such pulsatile stimulation of hormones up to the 11th hr (out of the entire 24 hr of treatment regime) to cultured Sc is the best time point for detecting the maximal augmentation of hormone-induced gene expression.

2. Material and Methods

2.1 Animals and Reagents

Pre-pubertal (12-day-old) Wistar rats (*Rattus norvegicus*) were obtained from the Small Animal Facility of the National Institute of Immunology (New Delhi, India). All animals were housed and used as per the national guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. Protocols for the experiments were approved by the Institutional Animal Ethics Committee (IAEC), National Institute of Immunology. Ovine (o) FSH, was obtained from National Hormone and Pituitary Program (NHPP), National Institutes of Health (NIH; Torrance, CA, USA). All other reagents, unless stated otherwise, were procured from Sigma Chemical (St. Louis, MO, USA).

2.2 Isolation of Sc

For each set of experiments, 30 testes from 15 male rats were pooled for the isolation of Sc. The procedure originally reported by Welsh and Wiebe[9], with minor modifications as previously described by us[10,11], was adopted for isolating Sc. Briefly, the testes were washed twice in Hanks' balanced salt solution (HBSS), decapsulated, and minced using a sterile blade. The tissue was digested in pre-warmed collagenase solution (6 mg collagenase per 30 mL HBSS) at 34 °C for 20 min in a shaking (120 oscillations/min) water bath. Small fragments of tubules were removed along with the supernatant. The supernatant containing small tubular fractions were pelleted at unit g for 5 min and the pellets were resuspended in HBSS and washed twice, pelleting at unit g to remove interstitial cells e.g. Leydig cells in the supernatant. The pellet was resuspended in 20 mL HBSS containing pancreatin (4 mg/20 mL). The digestion was carried out (3–6 min) at 34 °C until a large cell aggregate consisting mainly of Peritubular cells appeared, which was discarded by filtration. The remaining cells were pelleted (100 × g for 5 min), washed three times, and resuspended in a medium containing 1% fetal calf serum (FCS).

2.3 Long Term Sc Culture

On day 1 of culture, isolated Sc clusters were counted in an inverted phase contrast microscope (Nikon, DIAPHOT 300, under 20x magnification) and were seeded at a density of 0.5 × 10[5] cell clusters per well per mL, as previously reported by us[12]. The cultures were continued in DMEM-F12 HAM containing 1% FCS only for 24 hr in a humidified 5% CO_2 incubator at 34 °C. The next day, cells were washed with pre-warmed medium (DMEM-F12 HAM) and cultured further in serum replacement growth factor media (GF) containing 5 µg/mL sodium selenite, 10 µg/mL insulin, 5µg/mL transferrin, and 2.5 ng/mL epidermal growth factor. On day 3 of culture, residual Gc, if any, were removed by hypotonic shock by incubating Sc with 20 mM Tris-HCl (pH 7.4) for 3–5 min at 34 °C[13]. Sc were then washed twice in pre-warmed medium (DMEM-F12 HAM) to remove dead Gc and the cultures were continued further in GF media for the next 24 hr. The culture work plan is presented in Table 1.

Table 1. Summary of the experimental design of cultures and treatments

Day 1	Day 2	Day 3	Day 4
Sertoli cell isolation	Culture continued in 1% GF	Hypotonic shock to remove Gc and the culture continued in 1% GF	Treatments: i) Pulsatile FT (FTp) ii) Pulsatile control (Cp) iii) Constant FT (FTc) iv) Constant control (Cc) *Set-I*: Experiments were terminated at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr and 24.5 hr (Figure 1A), and saved in TRIzol. *Set-II*: Experiments were terminated at 9 hr, 9.5 hr, 10 hr, 10.5 hr, 11 hr,12 hr, 12.5 hr (Figure 1B), and saved in TRIzol.

The table shows the diagrammatic experimental flowchart of Sc culture for a period of 4 days. F12 + FCS = DMEM – F12 HAM containing 1% FCS which was added on day 1 only. GF = Growth factor media only. FT = FSH and T in combination. TRIzol = Tri-reagents for isolation of RNA.

2.4 *In vitro* Hormone Treatments

Set-I: On day 4 of culture, cells, were divided in 4 subsets: i. pulsatile FT (FTp), ii. pulsatile control (Cp), iii. Constant FT (FTc), and iv. Constant control (Cc), and were stimulated parallely.

- *Pulsatile group:* In the Pulsatile hormone exposure group (FTp), the GF media, during pulses, (for ½ hr only) contained hormones (50 ng/mL o-FSH and 10^{-7} M T in combination) and for following non-pulse time (2.5 hr), the GF medium was alone without hormones. For control of this treatment (Cp, pulsatile control group), Sc were exposed for ½ hr to the GF media alone without hormones and for following non-pulse time (2.5 hr), the GF media was cultured again without hormones. Several such pulses of treatment were given up to 24.5 hr (0 hr is the initiation of 1st pulse) periodically saving the cells at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr and 24.5 hr in TRIzol for the subsequent gene expression analyses as shown in Figure 1A and Table 1.
- *Constant group:* For constant hormone exposure group (FTc) cultured Sc were exposed continuously to hormones (50 ng/mL o-FSH and 10^{-7} M T in combination), until washed and treated with TRIzol. For control of this treatment (Cc, constant control group), Sc were exposed continuously to GF media alone without any hormone until washed and treated with TRIzol. These protocols were repeated for up to 24.5 hr, saving the cells at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr and 24.5 hr in TRIzol for the subsequent gene expression analyses as shown in Figure 1A and Table 1.

It is important to note here that, i) Sc were lyzed with TRIzol and stored at -80^0C for mRNA extraction at 6 hr, 12 hr and 24 hr (i.e. 2.5 hr after receiving the 2nd , 4th and 8th pulse of FT, respectively) where cells were in GF media without hormones at the time of termination, ii) Sc were saved in TRIzol at 6.5 hr, 12.5 hr and 24.5 hr (i.e. 1/2 hr after initiation of the 3rd, 5th and 9th hormone pulse, respectively) where cells were in GF+FT media at the time of termination. All experiments were repeated thrice on three different dates.

Set-II: In a separate experiment, cultured Sc were treated with pulsatile hormone (FT) for ½ hr followed by washing and re-incubation in GF media alone for 2½ hr, giving 5 pulses in entire $12^{1/2}$ hr time duration (0 hr was the initiation of the 1st pulse). The experiment was terminated at 9th hr (Sc received 3 pulses of FT and were in GF media at the time of termination), $9^{1/2}$ hr (Sc received 4 pulses of FT and were in GF+FT at the time of termination), 10 hr (Sc received 4 pulses of FT and were in GF media at the time of termination), $10^{1/2}$ hr (Sc received 4 pulses of FT and were in GF media at the time of termination), 11 hr (Sc received 4 pulses of FT and were in GF

media at the time of termination), 12 hr (Sc received 4 pulses of FT and were in GF media at the time of termination) and $12^{1/2}$ hr (Sc received 5 pulses of FT and were in GF+FT at the time of termination) by treating cells with TRIzol. For 9 hr and 12 hr termination time points there were 4 subgroups, e.g. Cc, or FTc or Cp or FTp. In rest of the termination time points there were only Cp or FTp. TRIzol samples were saved in −80 °C for mRNA extraction. The detailed experimental protocol and work plan of various treatments are given in Figure 1B and Table 1.

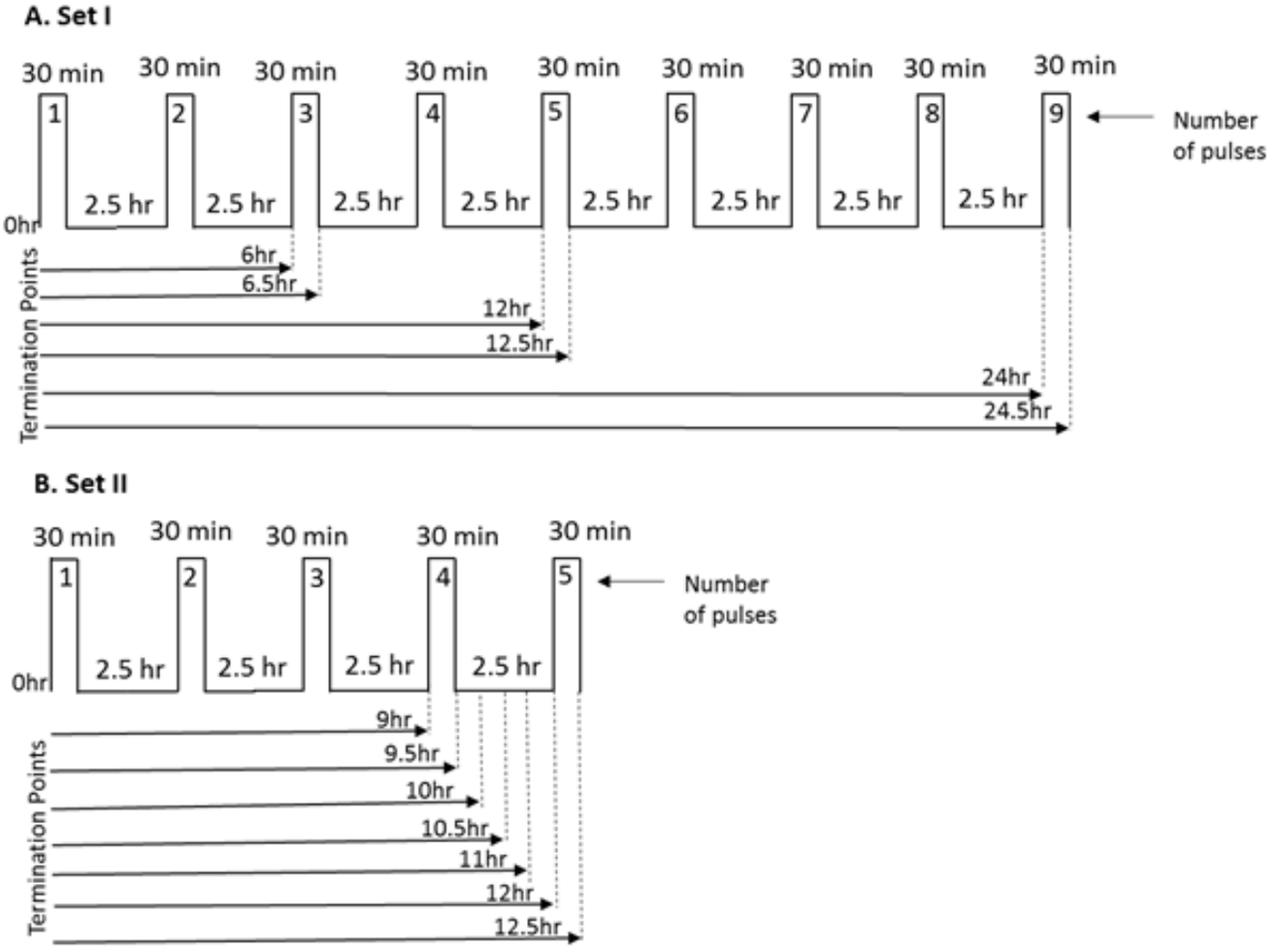

Figure 1. Schematic diagram of overview of cultures and treatments.

The Figure shows the *in vitro* pulsatile treatments of FTp and Cp from day 4 of culture. Sc were exposed for 30 min (pulse time) to GF media with hormones (FSH and T) in FTp and GF media alone without hormone in Cp. For both the groups, the medium was removed and replenished with fresh GF media without hormone for 2.5 hr (non pulse time). This was repeated for 24.5 hr (Figure 1A, for Set I) or 12.5 hr (Figure 1B, for Set II). For FTc and Cc, Sc were exposed continuously to GF media with hormones (FSH and T in combination) and GF media alone without hormone, respectively, until the termination points.

Analyses of the mRNA Expression of the Hormone Regulated Genes by Semi-quantitative RT-PCR

Phase separation to isolate total RNA from the TRIzol samples was carried out by adding 0.2 mL chloroform per mL TRIzol. The tubes were shaken vigorously by hand for 15 seconds and kept at room temperature for 2-3 min. The samples were centrifuged at 12,000 x g for 15 min at 4 °C. The upper aqueous phase was separated carefully and transferred to fresh tubes.. Isopropyl alcohol (propan-2-ol or 2-propanol) was added to each tube to half of the volume of TRIzol. The tubes were gently tapped and kept at room temperature for 15 min before centrifugation at 12,000 x g for 15 min at 4 °C. Next, the supernatant was decanted from each tube and RNA pellet in each tube was washed in 75% ethanol (equal volume of TRIzol per tube) at 9,500 rpm for 10 min at 4 °C. The RNA pellets were air-dried for 15min and the RNA was reconstituted in nuclease-free water (Ambion, USA). The RNA thus isolated was heated at 70 °C for 10 min and kept at 4 °C for 2 hr to get a homogenous solution. The isolated RNA was stored at −20 °C or −80 °C for respective short-term and long-term use. The total RNA (1 µg) isolated from each treatment group was first reverse transcribed using Reverse Transcription (RT) System (Promega Corp, USA) with AMV reverse transcriptase and oligo (dT)[15] for the single-strand cDNA synthesis. Subsequent PCR reactions (10 µL reaction volume) were carried out using 1 µL of the RT reaction as template for checking the expression profile of each gene. For each gene the number of PCR cycles was standardized to detect an acceptable expression level to confirm the findings. The list of gene primers used is given in Table 2.

2.5 Data Representation and Statistical Analysis

In all experiments, one treatment group comprised of 3−4 wells within one culture set. At least three such sets of cultures (performed on different dates) were used to interpret the data. Densitometric analyses of the gels for quantification of the gene expression levels were carried out using the *ImageJ software (ImageJ 1.33u)* provided by the National Institutes of Health, USA, in their public domain *http://rsb.info.nih.gov/ij*. Each gel photograph of RT-PCR is a representative of three independent experiments carried out on three different culture sets of each age group. One way *ANOVA* followed by Newman-Keuls Multiple Comparison test was conducted for group data where as non-parametric *Student's t-test* was performed between two groups for statistical analyses of the data.

Table 2. List of Primers

Gene	Sequence (5'-3')	Product size	Acc. No.
Inhibin β-B	F- AGCGCGTCTCTGAGATCATCA R- TCGGATGCGATGTCTGCTATC	458	NM_080771.1
Abp	F – ACAAGTTTCTGCATCCCTGGC R - TCCATCTTTGGTCCTTGGCTC	510	NM_012650.1
Scf	F –GCTTGACTGATCTTCTGGACAAG R -AACTGCCCTTGTAAGACTTGG C	505 (Soluble) 420 (Membrane-bound)	NM_021844.1
Cyclophilin A	F – TCACCATTTCCGACTGTGGAC R - ACAGGACATTGCGAGCAGATG	120	XM_341363.4

3. Results

3.1 Purity of Sc Culture

As previously described[12], Sc in culture formed confluent monolayers with purities of 95% as indicated by Oil Red O staining. Viability of cells on day 4 of culture was 98%. Although peritubular cell-specific alkaline phosphatase activity was present occasionally, contamination of peritubular cells was less than 2%. Leydig cells were absent, as indicated by the lack of 3β-HSD activity in the cultured cells (data not shown).

3.2 *Inhibin β-B* mRNA Expression

In Set I: The expression of *Inhibin β-B* mRNA (normalized against *Cyclophilin A*) was found to be increased gradually with time and exhibited heightened expression at 12 hr (0^{th} hr was the start time of the experiment). Augmentation of *Inhibin β-B* mRNA expression by FT_P was found highest among all treatments (C_C, FT_C, C_P) at this time point (Figure 2A). *Inhibin β -B* mRNA level declined at 12.5 hr although the transcription level in FT_C group was similar to that of the FT_P level at 12 hr (Figure 2A). *Inhibin β-B* transcripts were found to increase at 24 hr in all the four different treatments (Figure 2A). However, the 9 pulse at 24.5 hr mellowed down the gene expression in both C_P and FT_P groups, and where ever in constant treatments (i.e. both in C_C and FT_C) the transcript levels remained uniform (Figure 2A). The expression of internal control *Cyclophilin A* remained unaltered over the course of time of the experiment (Figure 2A).

In Set II: An appreciable augmentation of expression of *Inhibin β-B* mRNA under the influence of FT (FT_P over C_P) was observed at 9.5 hr (Figure 2B). However, no FT-mediated augmentation in gene expression was detected at 9 hr, irrespective of treatments (i.e. either constant or pulsatile), (Figure 2A). The augmentation detected at 9.5^{th} hr was lost at 10^{th} hr (Figure 2B) but recovered at 10.5 hr and 11 hr (Figure 2B). No further augmentation of expression of *Inhibin β-B* mRNA was detected at 12 hr, irrespective of treatments (i.e. either constant or pulsatile), and the level of transcripts remained uniform even after the 5 pulse (i.e. at 12.5 hr) (Figure 2B). The expression of internal control *Cyclophilin A* remained unaltered over the course of time of the experiment (Figure 2B).

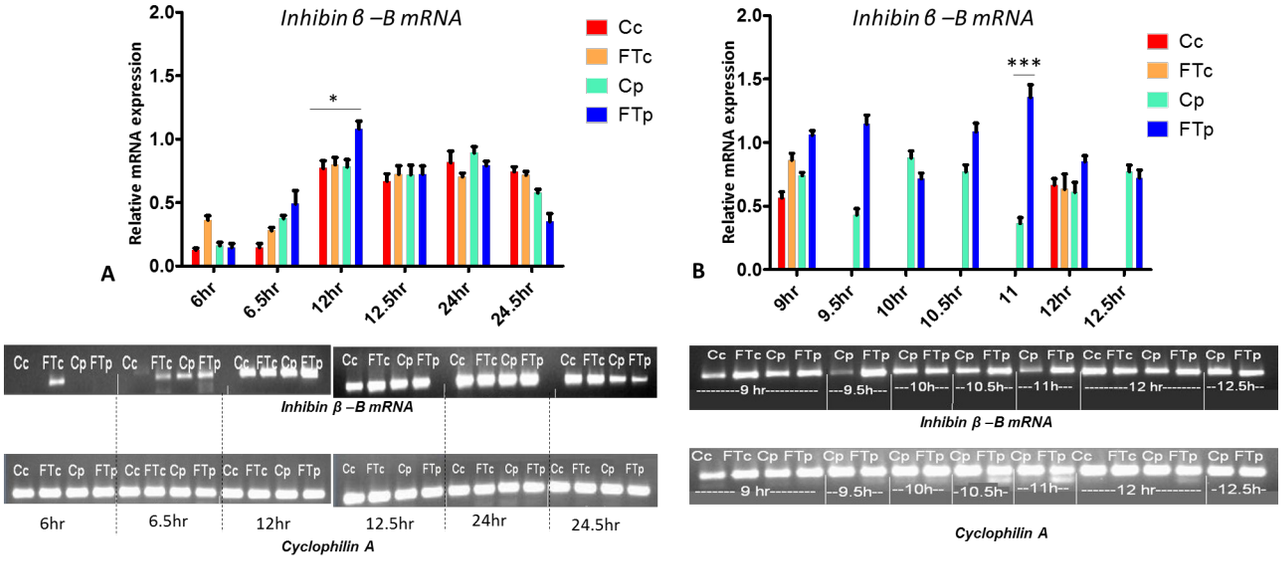

Figure 2. The effect of pulsatile hormonal treatment over conventional constant hormonal stimulation to Sc on *Inhibin β*-B mRNA expression. **(a)** *Inhibin β-B* mRNA expression at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr, and 24.5 hr. **(b)** *Inhibin β-B* mRNA expression at 9 hr, 9.5 hr, 10 hr, 10.5 hr, 11 hr, 12 hr, and 12.5 hr. C = GF medium alone, FT = GF medium with o-FSH (50 ng/mL) + T (10^{-7}M), Constant Control (Cc), Constant FT (FTc), Pulsatile Control (Cp), and Pulsatile FT (FTp).

Note: The gel picture is a representative of three sets of independent experiments and normalized with reference gene Cyclophilin A gene expression. Results are expressed as mean ± SEM of three independent experiments (Sc cultured on different calendar days). One way ANOVA followed by Newman-Keuls Multiple Comparison test was done for multiple groups in **A**, and non-parametric Student's t-test between FTc and FTp in **B.** Star (*) denotes statistical significance where *P<0.05, **P<0.01 and ***P<0.001.

3.3 *Abp* mRNA expression

In Set I: The expression of *Abp* mRNA (normalized against *Cyclophilin A* was also found to be increased gradually with time and reached the maximal level at 12 hr. Expression of *Abp* mRNA in FT_p group at this time point was found highest among all treatments (C_C, FT_C, C_p) at this time point (Figure 3A). A little FT mediated augmentation of *ABP* transcripts were detected at 6.5 hr in both the groups (constant and pulsatile treatments) Figure 3A. The mRNA level remained unaltered at 12.5 hr, except in FT_p group, where a down regulation was observed (Figure 3A). *Abp* transcripts (in all the 4 different treatments) were uniformly expressed at 24 hr at the level similar to that were observed at 12 hr in FT_p group (Figure 3A). The 9th pulse of FT (FT_p) at 24.5 hr inhibited the transcript whereas the transcript levels in rest of the treatments (i.e. C_C or FT_C or C_p) remained uniform (Figure 3A). Expression of internal control *Cyclophilin A* remained unaltered over the course of time of the experiment (Figure 3A).

In Set II: An augmentation of *Abp* mRNA under the influence of FT (FT_p over C_p) was observed at 9.5 hr (Figure 3B). However, a more prominent rise in *Abp* mRNA was detected at 9 hr only in constant treatment (i.e. rise in FT_C over C_C), (Figure 3B). The augmentation detected at 9.5 hr was maintained at 10 hr lost at 10.5 hr and recovered at 11 hr (Figure 3B). No further augmentation of *Abp* mRNA under the influence of FT was detected at 12 hr irrespective of treatments (i.e. either constant or pulsatile), and the level of transcripts remained uniform even after the 5th pulse (i.e. at 12.5 hr) (Figure 3B). The expression of internal control *Cyclophilin A* remained unaltered over the course of time of the experiment (Figure 3B).

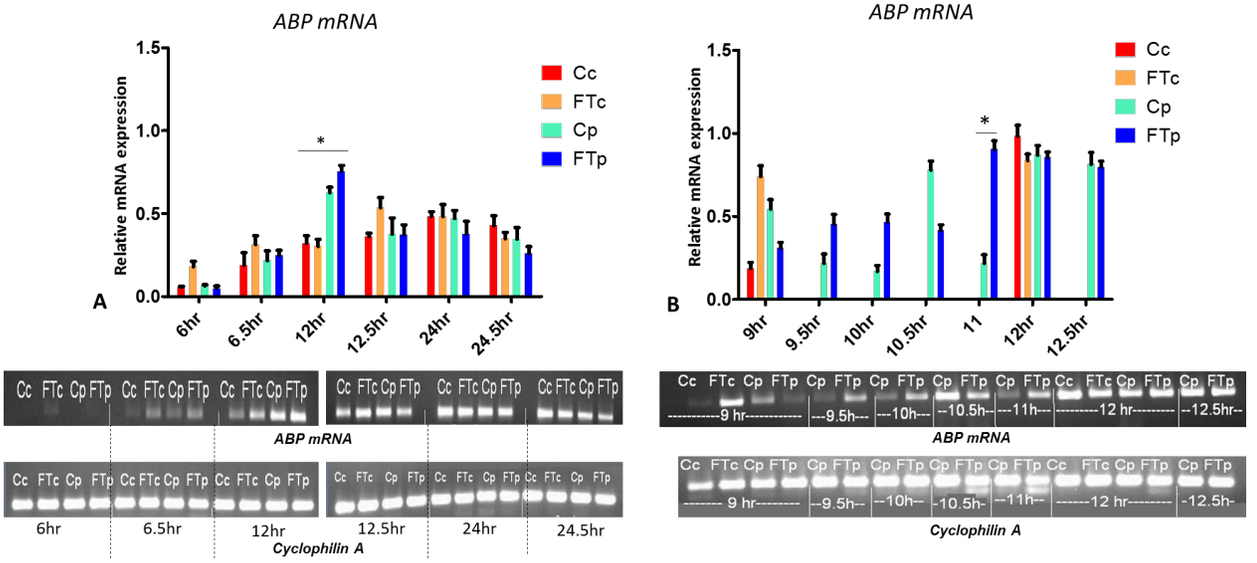

Figure 3. The effect of pulsatile hormonal treatment over conventional constant hormonal stimulation to *Sc* on *Abp mRNA* expression. **(a).** *Abp* mRNA expression at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr, and 24.5 hr. **(b)** *Abp mRNA* expression at 9 hr, 9.5 hr, 10 hr, 10.5 hr, 11 hr, 12 hr, and 12.5 hr. C = GF medium alone, FT = GF medium with o-FSH (50 ng/mL) + T (10^{-7}M), Constant Control (Cc),Constant FT (FTc), Pulsatile Control (Cp), and Pulsatile FT (FTp).

Note: The gel picture is a representative of three sets of independent experiments and normalized with reference gene Cyclophilin A expression. Results are expressed as mean ± SEM of three independent experiments (Sc cultured on different days). One way ANOVA followed by Newman-Keuls Multiple Comparison test was done for multiple groups in **A**, and non-parametric Student's t-test between FTc and FTp in **B**. Star (*) denotes statistical significance where *P<0.05, **P<0.01 and ***P<0.001.

3.4 *Scf mRNA Expression*

In Set I: The expression of *Scf* mRNA (the average expression level of both isoforms was normalized against *Cyclophilin A*) increased gradually with time and finally reached the peak expression at 12 hr. Expression of *Scf* mRNA in FT_P group at this time point was found highest among all treatments (C_C, FT_C, C_P) (Figure 4A). Both constant and pulsatile treatments of FT (FT_C or FT_P) induced a rise in *Scf* mRNA compared to the respective controls (C_C or C_P) at 6 hr and 6.5 hr time points (Figure 4A). A dramatic decline in *Scf* mRNA level was observed at 12.5 hr in all the treatment groups (either constant or pulsatile Figure 4A). This pattern remained sustained at 24 hr and 24.5 hr (Figure 4A). The expression of internal control *Cyclophilin A* remained unaltered over thecourse of time of the experiment (Figure 4A).

In Set II: The degree of augmentation was found to be highest at 11 hr (Figure 4B). Additional rise in *Scf* mRNA under the influence of FT was not detected at 12 hr irrespective of treatments (i.e. either constant or pulsatile), and the level of transcripts remained uniform even after the 5th pulse (i.e. at 12.5 hr) (Figure 4B). The expression of internal control *Cyclophilin A* remained unaltered over the course of time of the experiment (Figure 4B).

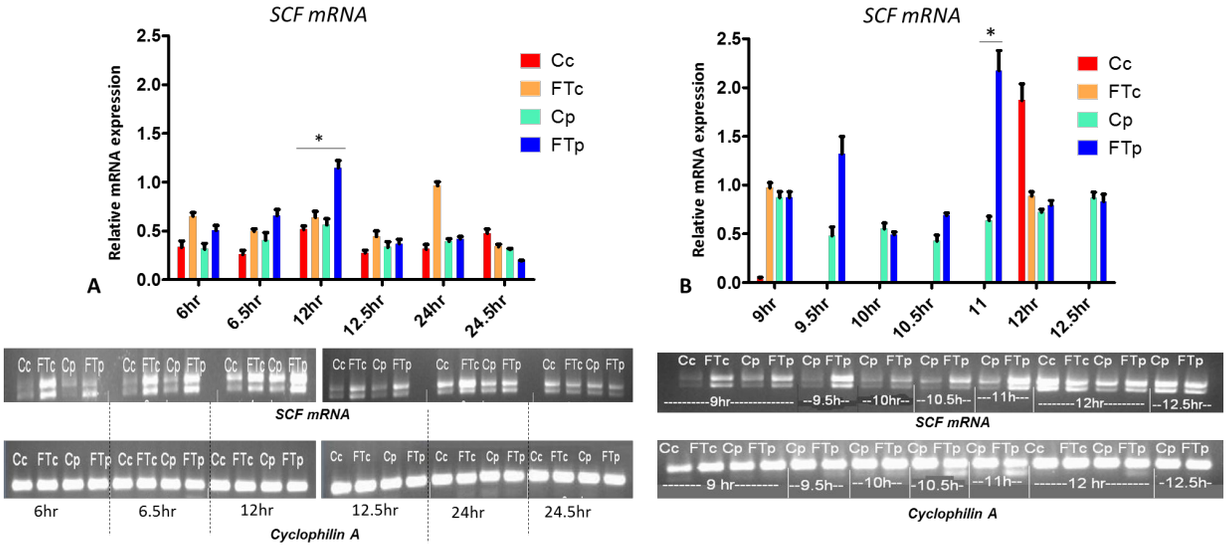

Figure 4. The effect of pulsatile hormonal treatment over conventional constant hormonal stimulation to *Sc* on *Scf mRNA* expression. **(a).** *Scf mRNA* expression at 6 hr, 6.5 hr, 12 hr, 12.5 hr, 24 hr, and 24.5 hr. **(b).** *Scf mRNA* expression at 9 hr, 9.5 hr, 10 hr, 10.5 hr, 11 hr, 12 hr, and 12.5 hr. C = GF medium alone, FT = GF medium with o-FSH (50 ng/ml) + T (10^{-7}M). Constant Control (Cc), Constant FT (FTc),Pulsatile Control (Cp), and Pulsatile FT (FTp).

Note: The gel picture is a representative of three sets of independent experiments and the average of two isoforms of Scf was normalized with the reference gene Cyclophilin A expression. Results are expressed as mean ± SEM of three independent experiments (Sc cultured on different calendar days). One way ANOVA followed by Newman-Keuls Multiple Comparison test was done for multiple groups in **A**, and non-parametric Student's t-test between FTc and FTp in **B**. Star (*) denotes statistical significance where *P<0.05, **P<0.01 and ***P<0.001.

4. Discussion

Primary culture of testicular cells is a potential tool to investigate the mode of actions of hormones (FSH and/or T) regulating spermatogenesis either alone or in combination[2,11,12,14–18]. Although traditionally constant exposure of hormones to cultured Sc is a routine practice, Sc are exposed to FSH and T (via LH) in a pulsatile manner rather than in a constant manner *in vivo*[19,20]. These hormonal pulses are generated by the unique secretary nature of FSH and LH in circulation from the pituitary which is further governed by GnRH pulses generated by

hypothalamus[20]. This typical pulsatile pattern of hormonal secretion is an important regulator to gain optimal hormonal function[20]. Furthermore, a majority of the *in vitro* studies have employed 3-isobutyl-1-methylxanthine (IBMX), a nonspecific inhibitor of phosphodiesterase (PDE) in culture, to obtain a better experimental readout[21]. Since IBMX is not naturally present in the body, it additionally introduces artificiality to the experimental system[22,23]. Therefore, hormone-induced gene expression data of Sc obtained from different laboratories have been found to be inconsistent[2,7].

We have recently developed a novel pulsatile hormonal exposure protocol for primary Sc cultures, unlike the conventional constant *in vitro* treatment, mimicking *in vivo* situation to assess consistent and effective hormone-induced gene expression pattern[8]. In the present study we have further pinpointed the exact treatment regime showing the maximum hormonal response in terms of gene expression by cultured Sc.

For this study, around 12-day old pre-pubertal rat Sc, reported to be most hormone responsive in culture as found by us earlier[12] were used. FSH and T were used in combination to stimulate cultured Sc in absence of IBMX[23] to avoid further artificiality with the system. Both pulsatile and constant hormonal treatments to Sc culture were run in parallel at different time points. Constant treatments (both control and FT) were given to the cells to compare their effect in terms of getting the maximum hormone-mediated transcription for a desired set of genes [namely, *Inhibin β-B, Abp* and *Scf* critically essential for sperm production] with that of the pulsatile treatments. Termination time points were determined in such a way that we could to detect the effect of the pulses in terms of hormone-mediated augmentation of gene expression (FT_p over C_p).

The data arising from *Set I* experiment (Figure 1A) suggest that the levels of transcripts of these three genes were found to be maximally elevated at 12 hr in FT_p group as compared to other treatment (C_C or FT_p or C_p) groups. After 12 hr time point, the transcripts were either maintained or decreased depending upon different parameters (treatments and / or genes).

Since the action of the FT pulse that augments gene expression was most prominent in between 2 consecutive pulses i.e. 4th pulse at 9 hr (0 hr was the start time of the experiment i.e. 1st pulse) and 5th pulse at 12 hr, we decided to investigate the expression profile of those genes in further detail in between 4th and 5th pulse i.e. in between 9 hr and 12 hr in *Set II* experiment (Figure 1B).

The expression levels of *Inhibin β-B, Abp* and *Scf* mRNAs were found to be augmented by FT after the 4th pulse at 9.5 hr. However, the expression levels were most elevated at the 11 hr, compared to that at 9.5 hr, 10 hr, 10.5 hr, and 12 hr. The extra 5th pulse from 12 hr to 12.5 hr however failed to augment the gene transcription any further.

The down regulation of gene expression observed in between 9 hr and 12 hr, especially at 10 hr and / or 10.5 hr, might be due to two possible signaling outcomes. First, an extended activation of CREB-CREM might lead to generation of inducible cAMP early repressor (ICER) and subsequent silencing of *Inhibin β-B* and *Scf* genes at the promoter level. Second, alternative or simultaneous up-regulation of ID repressors would lead to the suppression of upstream stimulatory factor (USF)-mediated transcription from the E-box, a conserved sequence present on the promoter of *Abp*. The role of ICER in the regulation of FSH-responsive gene expression is well established[2]. In cultured Sc, FSH stimulates the highest ICER expression after 4 hr, and the ICER protein levels remain elevated up to 24 hr. Functional studies in primary rat Sc show that ICER increases rapidly upon FSH stimulation, indicating that it may be involved in rapid down-regulation of FSH responsive transcripts. This repression is due to binding of ICER to CRE-like sequence in the FSH responsive gene promoter[2].

5. Conclusion

Taken together, the present study has established to a great extent the efficacy of our pulsatile hormonal treatment protocol to primary Sc cultures in order to demonstrate the highest hormone-induced gene expression. In particular, this study suggests that such pulsatile hormonal exposure to Sc cultures is most effective in terms of hormone-induced gene expression at 11 hr of the entire 24 hr of pulsatile treatment regime. In future, this new protocol of hormonal stimulation to Sc will be a better choice for detecting the hormonal impact on gene expression more accurately and, therefore, may substantially improve the *in vitro* validation of hormone-responsive candidate genes crucial for spermatogenesis.

6. Abbreviation

FSH: Follicle stimulating hormone, T: Testosterone, Sc: Sertoli cells, Gc: Germ cells.

7. Competing Interests

The authors declare that they have no competing interests.

8. Acknowledgments

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- **Author Contributions**

IB and SSM designed the research. IB performed all the experiments. IB and HS analyzed the data and wrote the paper.

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