Original Research
ISSN (Print): 0971-913X
ISSN (Online): 2455-1244
J Endocrinol Reprod. *Vol 27(2), 91-98, June 2023, DOI: 10.18311/jer/2023/34311*

The Effect of Methallibure (ICI Compound 33, 828) on Folliculogenesis and Steroidogenic Potential of Developing Ovary in the Lizard, *Calotes versicolor (Daud.)*

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Abstract

In vertebrates, it is well established that gonadotropins are crucial for vertebrate ovarian development and its endocrine functions. The current investigation envisages determining how methallibure affects the histoanatomical structure and steroidogenic capacity of the developing ovary of 10-day-old female hatchlings of *Calotes versicolor.* The eggs were collected from gravid females during the breeding season and incubated at a female-producing temperature of 31.5±0.5 ^{o}C till hatching. Hatchlings were fed with live termites. Intraperitoneal injections of 6µg of methallibure in 0.05ml of 0.7% saline were injected into 10-day-old female hatchlings on alternate days for 21 days. The appropriate saline-treated hatchlings served as control. The results reveal that methallibure significantly reduces the number of oocytes ($P<0.02$) and primordial follicles, decreases their diameter, and increases the number of previtellogenic atretic follicles ($P<0.001$) with concomitant suppression of the progression of ovarian development when compared with that of baseline control. Three types of atretic follicles were noticed. The histoenzymatic activity of Δ^5-3β-hydroxysteroid dehydrogenase in the ooplasm of the follicle and the stromal region showed a decreased intensity. The downregulation of steroidogenesis is probably due to the deprived secretion of gonadal steroids which disturbs the negative feedback mechanism because of the action of the drug at the hypothalamic-hypophyseal-ovarian axis. The present study unveils that methallibure acts as an antigonadotrophic drug altering the synthesis/secretion of gonadotrophin by hypophysis. It is inferred that methallibure induces an anti-gonadotropic effect in the newly hatched hatchlings probably by quenching the pituitary gonadotropins.

Keywords: Atresia, Hatchlings, Lizard, Methallibure (ICI, 33,828), Ovary, Δ^5-3β-HSD

1. Introduction

Methallibure, ICI, 33,828 (1-methylallyl thiocarbamoyl-2-methylthiomoylhydrazine) a nonsteroidal compound causes gonadal regression in vertebrates by specifically obstructing the synthesis/release of gonadotropins at the hypophysial level[1,2]. The anti-gonadotropic effect of methallibure is extensively investigated in several fishes[3-9], amphibians[10-12], birds[13] and mammals[1,2,14]. Most studies in different vertebrate classes have reported that the synthesis and/or release of gonadotropins is blocked by methallibure. Coincidentally, there are only a few studies on the impact of methallibure on the testes and pituitary in adult reptiles[15,16]. As far as the authors are aware, there are nil data regarding the impact of methallibure on the ovaries of adult or young reptiles.

The two pituitary gonadotropins have distinct functions in controlling ovarian function in eutherian

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Article Received: 19.12.2022 Revised on: 14.06.2023 Accepted on: 01.07.2023

and marsupial mammals. While Luteinizing Hormone (LH) promotes ovulation and steroidogenesis, Follicle-Stimulating Hormone (FSH) controls oogonial proliferation, oocyte development, and differentiation. It is still unclear, nevertheless, whether one or two gonadotropins exist in many reptile taxa. According to fractionation analysis, turtles and alligators exhibit two separate gonadotropins that resemble mammalian LH and FSH[17,18]. The purification and characterization of pituitary gonadotropins from several squamate families, on the other hand, reveal the existence of a single gonadotropin that combines FSH- and LH-like activity[18,19]. To date, the cDNA for either FSH or LH, but not both, has been cloned from squamate species.

Some adult species of reptiles have been used to evaluate the impact of mammalian gonadotropins[19–22]. Unfortunately, hardly a few studies are divulging the role of gonadotropins in hatchlings or immature ones[23-25]. Additionally, compared to representatives from the temperate zone, our comprehensive knowledge of how the Hypothalamo-Pituitary-Gonadal (HPG) axis controls ovarian endocrine activity is very meagre in tropical reptiles.

Therefore, research into how gonadotropins affect oocytes and primordial follicles' development and their maintenance during the early phases of gonad development in reptiles is necessary. It is well established that hypophysectomy offers a way to examine the gonads' dependence (or lack thereof) on gonadotropins. Nevertheless, hypophysectomy deprives the gonads of gonadotropins besides interrupting the entire hormonal milieu. In addition, hypophysectomy in newly hatched hatchlings is technically quite hard as their rearing itself is challenging. Chemical inhibition of pituitary gonadotrophins can be accomplished using many compounds, but most of them are steroids and they impede the feedback pathways. Hence, a nonsteroidal and non-hormonal compound, methallibure, was employed for the suppression of pituitary gonadotropins in newly hatched hatchlings of *Calotes versicolor* that evinces a unique TSD pattern. The 10-day-old hatchlings were chosen for the present investigation because the debut of primordial follicles occurs during this period. Hence, the present investigation aims to know the impact of methallibure on the 10-day-old hatchlings' ovary concerning i) proliferation of oogonia and folliculogenesis, ii) development and maintenance of oocytes and follicles and iii) action on granulosa cells in the lizard, *C. versicolor.*

2. Materials and Methods

Calotes versicolor, polyautochronic, multicultured lizard, which has an extended breeding phase (May to October). It retains eggs in the oviduct for about a fortnight that depends on environmental conditions, especially temperature and rainfall. Normally, it lays about 6-32 eggs in a clutch. Before the oviposition, the development of the embryos from stages 1 through 26 takes place when the eggs are still there in the oviduct. Oviposition occurs at embryonic stage 27 and hatching at stage 42. During the earlier part of the development (stages 27-35), the interval between two consecutive stages is about 2-3 days and for the later part (stages 36-42) is 4-5 days while it requires 60-75 days for hatching[26].

2.1 Collection and Incubation of Eggs

Gravid female lizards were caught from the regions around Dharwad (15^0, 170'N, 75^o, 30'E), Karnataka, India. They were housed in a reptile dwelling (20' X 20' X 10') with wire mesh on all sides for quarantine (15–20 days). Food (grasshoppers/cockroaches/dragonflies) and water were supplied *ad libitum*. The eggs were collected from gravid females (enlarged abdomen indicating the presence of eggs), randomly assigned, padded with moist cotton, and incubated at 31.5±0.5 ^{o}C (female-producing temperature). The incubation temperature was monitored ensuring the temperature fluctuation did not exceed 0.5^{o}C while maintaining a relative humidity at 62%.

When necessary, water was sprinkled over the cotton to maintain constant optimum moisture. Petri dishes were rotated daily within incubators to ensure uniform temperature distribution and to avoid the potential influence of undetected thermal gradients on the sex determination of developing embryos. Incubation methods, as described elsewhere[27-29]. The eggs were incubated from oviposition (stage 27) to hatching (stage 42). The hatchlings were housed in a glass aquarium (75cm x 37.5cm x 50cm) separately and fed regularly with live termites/baby grasshoppers and supplied water *ad libitum*.

Of the total fifteen 10-day-old hatchlings, five (5 nos.) were weighed, and the ovaries were dissected out and subjected to histology as initial control. The remaining

hatchlings were separated into two groups consisting of five hatchlings each. Group I was given 0.05mL of 0.7% saline that acts as baseline control and Group II received 6 µg of methallibure in 0.05ml of 0.7% saline. For 21 days, intraperitoneal injections were administered on alternate days. A day after the last injection, all hatchlings were sacrificed and their weights were recorded. The ovaries were subjected to Δ^5-3β HSD enzyme activity and were processed for the following histometric and routine histological analysis:

1. Oocyte and follicle counts were noted from every fifth section of the ovary- A follicular kinetics analysis.
2. The frequency of types of atretic follicles and their status were recorded.

2.2 Histology

Immediately after being sacrificed, the ovaries were dissected out fixed in Bouin's fluid, dehydrated in graded concentrations of ethanol (10%-100%), clarified in benzene, and embedded in paraffin. Serial sections of 5µm were cut on a rotary microtome (Leica RM 2025, Germany), stained with Harris-Haematoxylin and counterstained with eosin and mounted with DPX mountant.

2.3 Enzyme Histochemistry

For the demonstration of Δ^5-3β HSD enzyme activity, the ovary was frozen over dry ice vapour, and 14-16 µm thick sections were cut in a "Pearse-Slee" cryostat at -18°C. After thawing at room temperature, the sections were incubated at 37°C in appropriate media. The Pregnenolone (3β-hydroxypregnen-5-ene-20-one) and Dehydroepiandrosterone (3β-hydroxy androst-5-ene-17-one) were used as substrates (1mg/ml). To prepare the incubation media, the steroids were dissolved in a drop of dimethyl formamide. The β-nicotinamide adenine dinucleotide (1.5mg/mL) was used as a co-enzyme and nitro blue tetrazolium (0.5 mg/mL) as the final hydrogen acceptor, all including substrates dissolved in 0.2M phosphate buffer at pH 7.4.

The preparation of the incubation media and other details of the protocol were followed as described previously[27-30]. The intensity of enzyme activity was characterized depending on the quantity of diformazan granule deposition and by visual grading of reaction [from traces (±) to intense (++++)]. The analytical grade chemicals were procured from Sigma Chemical Company and were used as received. All the experiments on animals were conducted following the protocol of CPCSEA and the Institutional Animal Ethical Committee (IAEC) No. 639/GO/02/a/CPCSEA guidelines of Karnataka University, Dharwad.

3. Observations

3.1 Initial Control

The ovary was tiny, morphologically seeming slightly elongated, thick in the centre, and tapering at both ends. Foldings were observed in the periphery of the ovary. Observed under the binocular microscope, the ventral surface of the ovary consisted of irregular areas consisting of shallow grooves where compactly arranged oogonia and oocytes were seen. Oogonia proliferates by mitosis, subsequently developing into the primary oocyte. These primary oocytes arrest division at the diplotene stage in the prophase of the first meiotic division. The short columnar epithelial cells surrounded the oocyte of the primordial follicle. The ovary's stromal area is comprised of cords of cells having round to oval nuclei and abundant connective tissue and blood spaces (Figure 1A).

3.2 Saline Control

The saline-treated one-month-old hatchling's ovary compartmentalized into germinal bed, stromal, and intrastromal regions. The germinal bed of the ovary is covered by a surface epithelium and consisted bunch of oogonia. There were numerous "naked" oocytes observed towards the stromal region (Figure 1B). At the periphery, there were a few small primordial follicles (40 µm), while larger follicles (75 µm - 80 µm) were noticed closer to the stromal region. Large follicles were covered by 3 types of granulosa cells viz., small, intermediate, and large round cells (the future pyriform cells) whereas small follicles were surrounded by a single layer of flattened epithelial cells (Figure 1C). In comparison to large round cells, the small and intermediate cells were more numerous. Zona pellucida of large follicles composed of a non-striated, homogenous, thin membrane. One to two layers of fibroblast-like thecal cells formed a thin thecal membrane (Figure 1C). Except for a few atretic follicles, the majority of the follicles were normal. The small stromal area consisted of a few interstitial cells, connective tissue, and many blood spaces.

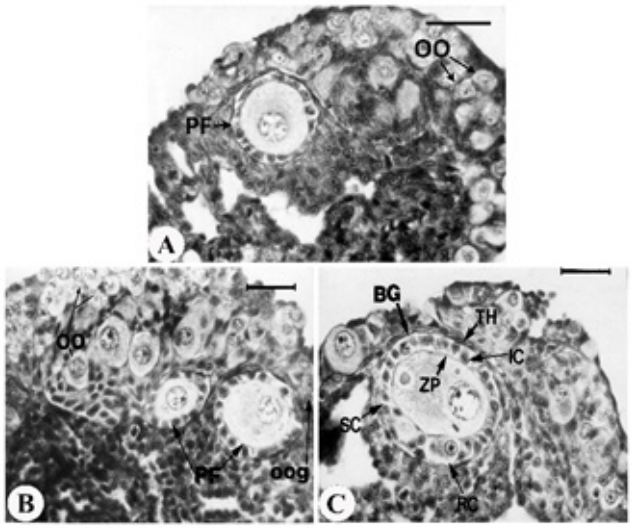

Figure 1. **(A)** T.S. of the ovary of Initial control, a 10-day-old hatchling of *C. versicolor* showing small Primordial Follicles (PF) and Oocytes (OO) at different stages of meiotic prophase-I. Scale bar – 30 µm. **(B)** T.S. of the ovary of saline control, a one-month-old hatchling of *C. versicolor* showing a few developing Primordial Follicles (PF) and many Oocytes (OO). Scale bar – 30 µm. **(C)** T.S. of the ovary of saline control, a one-month-old hatchling showing details of one large follicle (BF) revealing polymorphic granulosa consisting of Small Cells (SC), Intermediate Cells (IC) and Round Cells (RC). TH- Theca, ZP – Zona pellucida. Scale bar – 30 µm.

3.3 Methallibure-Treated Hatchlings

Compared to the control group, the treated group's gonadosomatic index did not significantly reduce (Figure 3A). Histology of the ovary disclosed atrophy with significant depletion in the oocytes' count (Figure 3B; $P < 0.02$) and follicular diameter (Figures 2A and B). Many degenerating oocytes were found. Comparing the atretic follicles' count of control (Figure 3C; $P < 0.001$) there was a significant increase in that of the treated group (Figure 2 B to D). Developing follicles exhibited extensive atresia. The observed 3 types of previtellogenic atretic follicles are as follows:

Type - I

Pycnotic nuclei were present in the granulosa cells of the previtellogenic follicles. The granulosa layer along with the ooplasm moved inside after getting detached from the follicular wall /thecal layer (Figure 2A). The zona pellucida was not lucid and the single layer of granulosa was interrupted at certain regions.

Type - II

Herein, ensuing the collapse of the zona pellucida, granulosa cells invaded the ooplasm and 2-3 layers of granulosa comprising of small cells contained pycnotic nuclei (Figure 2B). The nucleus of granulosa cells appeared to contain crumpled or degenerating chromatin material.

Type - III

The bigger follicles (75-80 µm) of the treated group exhibited Type-III atretic follicles.

The vacuoles were noticed in the ooplasm during the early stage of atresia. The granulosa layer got detached from the thecal layer at certain regions (Figure 2C). During the later stage of atresia, the oocyte was encircled by a group of cells. The granulosa layer and thecal layer could not be distinguished (Figure 2D). Zona pellucida was not distinctly visible.

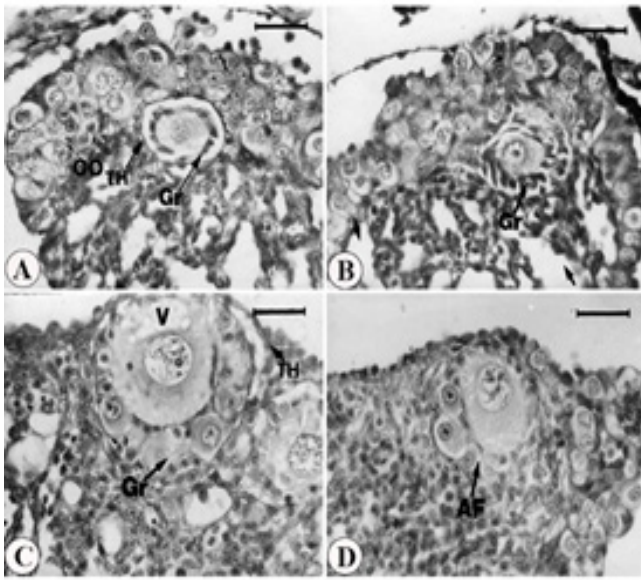

Figure 2. **(A)** T.S. of the ovary of a methallibure-treated hatchling of *C. versicolor* showing Type – I atretic follicle. Gr – Ganulosa, TH - Theca, OO – Oocytes. Scale bar – 30 µm. **(B)** T.S. of the ovary of methallibure-treated hatchling showing Type – II atretic follicle. The stromal region shows lacunae (arrow). Gr – Ganulosa. Scale bare – 30 µm. **(C)** T.S. of the ovary of methallibure-treated hatchling showing Type–III atretic follicle at the early stage of atresia. Gr – Ganulosa. Scale bar – 30 µm. **(D)** T.S. of the ovary of methallibure-treated hatchling showing Type – III atretic follicle at an advanced stage. AF – Atretic follicle. Scale line – 30 µm.

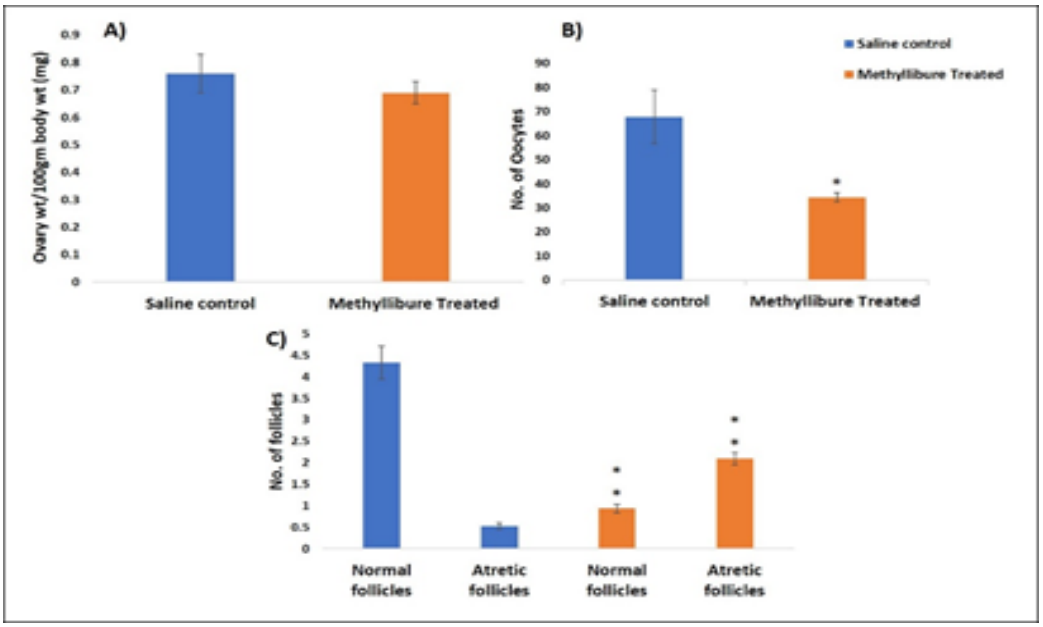

Figure 3. **(A-C)** Effect of methallibure treatment on morphometric and follicular kinetics of the ovary in *C. versicolor*. **(A)** Graph showing the ovary weight/100g body weight. **(B)** No. of oocytes, **(C)** No. of follicles (atretic and normal) in the saline control and methallibure treated groups. There was no reduction in the Gonadosomatic Index (GSI) of the methallibure-treated group. There was a significant reduction in the number of oocytes and an increase in the number of atretic follicles in the methallibure-treated group when compared with saline controls. *: $p<0.02$, **: $p<0.001$.

3.4 Histochemical Observations

A progressive decline in the expression of Δ^5-3β-HSD enzyme activity was noticed in the treated ovary when compared to that of the control (Table 1). Owing to the presence of numerous atretic follicles, and the intensity of enzyme activity being very feeble, it was very difficult to visualize the normal follicle of the treated ovary. The ovarian expression of Δ^5-3β-HSD enzyme activity decreased in the previtellogenic follicles also (Table 1).

4. Discussion

Administration of Methallibure (ICI,33,828-derivative of dithiocarbamoyl hydrazine) is known to induce gonadal suppression/repression by inhibiting gonadotropin output from hypophysis in several vertebrates. Following the pioneering work of Paget *et al.*, in 1961[1] on Methallibure in rats, extensive studies have been carried out on this drug in fish and mammals. Concerning fish, this compound is known to interfere with oogenesis, block

Table 1. The intensity of 3β-HSD activity in the developing ovary of the lizard, *Calotes versicolor*

Group	Stromal region /interstitial cells	Oocytes and Ooplasm of previtellogenic follicle
Control (5)	+ ±	+ ±
Methyllibure Treated (5)	±	±

Intensity of enzyme activity is visually graded from (±) to (+±); (±) denotes trace activity, (+±) a minimum activity.

vitellogenesis and spermatogenesis and induce atresia of yolk oocytes by inducing inhibition of hypothalamic-hypophyseal-gonadal activity[3-9,31]. Methallibure blocks the gonadal differentiation[4] and vitellogenesis by inducing atresia of oocytes, inhibits ovarian development and causes interruption of gestation in juvenile guppy, *Poecilia reticulata*[8]. The interdiction of Gonadotropin-II (GTH-II) and testosterone production are reported in *Clarias batrachus*[31]. Gonadotropins promote ovarian development, ovulation, oviductal growth, vitellogenesis, and steroidogenesis in adult female reptiles[20-22].

The present study of methallibure treatment for a 10-day-old hatchling of *C. versicolor* resulted in a noteworthy decrease in the oocytes' count ($P < 0.02$) and diameter of follicles. The observed results divulge that gonadotropins are crucial for the proliferation of oogonia and oocyte maintenance in the hatchlings. An earlier report from our laboratory on histoenzymological study on the development of ovary related to oogenesis, folliculogenesis and steroidogenesis revealed the onset of the primordial follicle in 10-day-hatchlings suggesting albeit indirectly possible utilization of gonadotropic hormones[27] and confirms these results. Further, the ovary contained extensive atretic follicles where 3 types of follicular atresia were noticed. The observed findings point towards the impediment in the release of gonadotropins from the hypophysis as reported across the vertebrates[1-14]. In the current study, the steroidogenic potential of the methallibure-treated ovary of 10-day-old hatchlings diminished as indicated by the downregulation Δ^5-3β-HSD enzyme activity which was very difficult to visualise. The Δ^5-3β-HSD is a key enzyme which catalyzes the oxidative transformation of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, a key step involved in the synthesis of bioactive steroid hormones[22]. The observed down-regulation of ovarian steroidogenesis is probably due to the deficiency of secretion of gonadal steroids which disturbs the negative feedback mechanism because of the action of the drug at the hypothalamic-hypophyseal-ovarian axis. These findings demonstrate that methallibure causes regressive alterations and decreases the ovary's capacity to produce steroids, possibly due to the inhibition of gonadotropins' release from the hypophysis subsequently interfering with the steroidogenic pathway.

This confirms that methallibure acts as an anti-gonadotropic compound in the hatchlings of *C. versicolor* and is analogous to findings in several other vertebrates[1-14]. The administration of methallibure to five-day-old pigeon hatchlings, *Columba livia,* resulted in a reduction in Δ^5-3β HSD and G-6-PDH activity in the Leydig cells[13].

Earlier studies on the immature reptile, *Agama agama* disclosed that hypophysectomy did not cause atresia in small previtellogenic follicles suggesting that maintenance of previtellogenic follicles may be independent of gonadotropins[32]. However, FSH administration to juvenile/immature lizard, *Anolis carolinensis* is observed to enhance the primary oocytes' and primordial follicles' count in the ovary and induces ovulation[19,23]. Further, hypophysectomy in adult *A. carolinensis* leads to a decrease in the number of primary oocytes and primordial follicles[19,21]. Besides, in this species administration of FSH leads to augmented growth of follicles of different sizes and recruitment of follicles within the germinal bed and their movement, one at a time in into the stromal region has been noticed[19,21,23,24]. Also, in female juvenile lizards, *Eumeces obsoletus,* FSH stimulates germ cell proliferation and steroidogenesis[25]. These findings suggest that the formation as well as maintenance of folliculogenesis in lizards are gonadotropin-dependent and are under the control of the Hypothalamus–Pituitary–Gonadal (HPG) axis.

The present investigation may be of use in understanding how ovarian activity is regulated by the HPG axis and other hormones in juveniles/hatchlings and may eventually reinforce the successful management of reptilian species, both *in situ* and *ex-situ*.

The current study divulges that gonadotropins are required for the proliferation of oogonia and early folliculogenesis, supporting the view of Jones *et al.,*[21,23] wherein the forming and maintenance of primary oocytes and primordial follicles are gonadotropin-dependent in hatchlings as well as in adult lizard, *C. versicolor*. It is inferred that methallibure induces an anti-gonadotropic effect in the newly hatched hatchlings probably by quenching the pituitary gonadotropins. Before drawing any conclusion regarding the molecular and physiological mechanisms underlying methallibure's mode of action, considerable work needs to be done.

5. Acknowledgements

LSI gratefully acknowledges the financial support from the DST-SERB Grants, New Delhi under DST Major

Research Project (CRG/2018/004301 dtd. 22.03.2019). RMH thanks the DST-SERB for the award of JRF.

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