Artykuł przeglądowy *Review*

Downregulated canine testis and recrudescence of testicular function following abolition of downregulation

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Summary

The method of choice for the downregulation of canine testicular function is the use of slow-release GnRH-agonist implants. Clinical efficacy has well been demonstrated, and basic information on the underlying mechanisms of action has been obtained. As reviewed in the present paper, the treatment-induced decreased availability of LH is followed by the downregulation of the steroidogenic apparatus and – restricted to the protein level – of androgen receptor expression. A downregulated testis is distinctly different from a juvenile one and rather resembles the situation observed in testes of seasonally breeding animals out of season. Following the abolition of downregulation, the onset and recrudescence of testicular function is a rapid process. Full steroidogenic capacity is regained within 3 weeks, and spermatogenesis enters the spermatogenic cycle leading to pre-treatment ejaculate quality.

Keywords: dog, testis, downregulation, recrudescence

Definition of downregulation

The downregulation of testicular function implies that endocrine and germinative testicular functions are lost or at least significantly inhibited with the testis still remaining *in situ*. Such a situation will result from the removal of gonadotropic support and may be accomplished by interfering with the availability of gonadotropin-releasing hormone (GnRH) and/or of LH, formerly known as interstitial cell-stimulating hormone (ICSH) in the male animal. Both hormones are key hormones in the neuroendocrine control of testicular function (Fig. 1).

The abolition of endogenous gonadotropic support may be achieved by reducing the frequency of GnRH pulses by progestin treatment (15), by active immunization against GnRH or by treatment with GnRH antagonists or agonists to interfere with GnRH binding to pituitary cells (4, 22, 29).

Regardless of the approach, effects on testicular function have been shown to be reversible. However, as regards efficacy, there are certain limitations. Progestins had only a limited effect on the release of LH and on semen quality (5), immunization against GnRH generally failed to generate long-lasting effects, and the use of GnRH-antagonists is still at a preliminary stage (4, 8, 18).

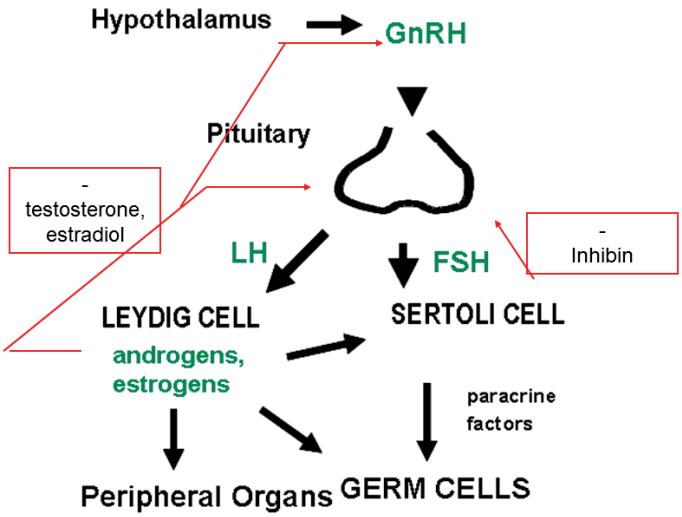

Fig. 1. Neuroendocrine control of male reproduction

Thus, up to now, the approach of choice for a successful and reversible block of testicular function that meets the demands of veterinary practice has been the

use of slow-release implants containing a GnRH agonist. Following an initial stimulation, the application of slow-release implants leads to the downregulation of pituitary GnRH receptors (21) resulting in a significantly reduced gonadotropin secretion and the loss of testicular activity. Similar effects were observed when buserelin acetate (22), azagly nafarelin (20) or deslorelin (17, 28) had been used as active ingredients. However, the onset and duration of downregulation may vary significantly between dogs (13). At present, deslorelin is available as a slow-release implant for use in small animal medicine [Suprelorin® (Virbac)] with the official indication reading: "induction of temporary infertility in healthy, entire, sexually mature male dogs and ferrets". However, as our own studies have clearly shown, all androgen-based clinical disorders are in fact indications for the use of this drug (13).

The course of downregulation

Changes in testosterone concentration in peripheral plasma are a clear indicator regarding the loss of testicular endocrine function. As demonstrated in a preclinical study on Beagle dogs with a slow-release implant containing azagly nafarelin as the active ingredient (20), testosterone concentrations increased rapidly after implantation, remained above pre-treatment values for about 4 days, and started to decrease after day 6. The mean time to reach basal levels (</= 0.1 ng/ml) was 17.5 ± 8.4 days (Fig. 2). Clearly this loss of steroidogenic capacity is related to the reduced availability of LH, as indicated by the significantly reduced area under the AUC curve shown in Fig. 3. In parallel to the reduced availability of testosterone, the size of testis decreased by 80% to the minimum in week 17 after implant injection, and the size of the prostate was decreased by 46% in week 5. These effects were confirmed in a clinical study on 53 male dogs of different breeds, ages and health status, which showed their individual responses to treatment. Thus, full downregulation lasted from 6 to over 22 months in 51 dogs, one dog did not respond, and another showed a transient downregulation only. The mean testicular and prostatic sizes decreased significantly, and the effect on the prostatic size was more pronounced in dogs with benign prostatic hyperplasia (13). Basically, these observations agreed with those by Junaidi et al. (16, 17), who used deslorelin implants.

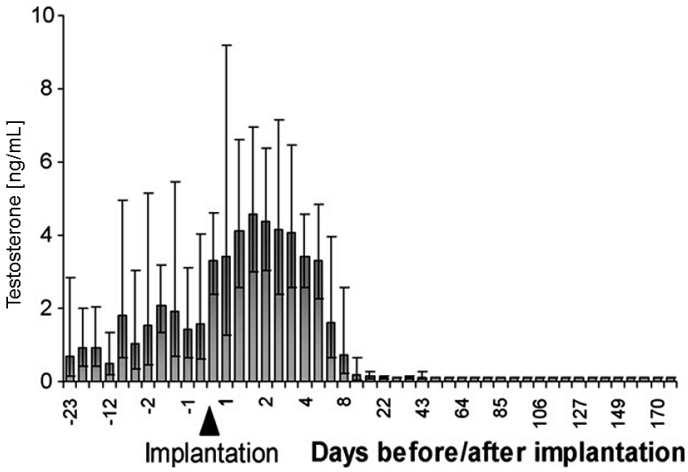

Fig. 2. Testosterone concentrations (Xg x DF$^{\pm 1}$) prior to and after the implantation of a slow-release implant containing 18.5 mg of the GnRH agonist azagly-nafarelin (according to (20))

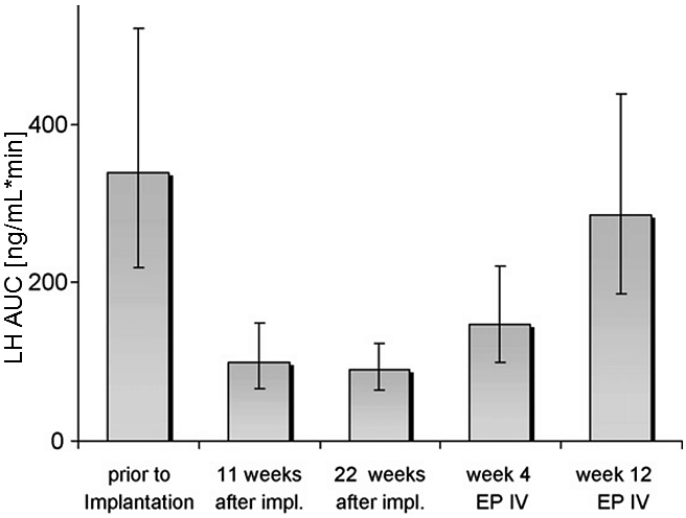

Fig. 3. Availability of LH (Xg x DF$^{\pm 1}$) as indicated by the AUC prior to and 11 weeks after the implantation of, as well as 4 and 12 weeks after the removal (EP IV) of a slow-release implant containing 18.5 mg of the GnRH agonist azagly-nafarelin (according to (20))

Following treatment with the implant containing azagly nafarelin, ejaculates could be collected for up to 5 weeks. While the volume decreased with time, sperm concentration was variable, showing an increase or a decrease with a tendency for an increased percentage of spermatozoa with abnormal morphology (19).

The downregulated testis

Apart from the fact that the downregulation of testicular function is characterised by the loss of or, at least, a significant reduction in testosterone secretion, only little information has been published on the status of the downregulated testis. Clearly, the state of downregulation achieved does not depend on the type of the GnRH agonist applied if used in the recommended dose (9). In the downregulated testis, the most developed germ cells observed were spermatogonia (22) and few primary spermatocytes (10, 12) (Fig. 4A). As shown by Goericke-Pesch et al. (10), there were significant effects on the tubule area (μm^2), which decreased by around 74%, on the area (μm^2) of Leydig cell nuclei, which decreased by around 35%, on the total number of spermatogonia, which decreased by around 50%, and on the ratio of "area tubular compartment" to "area interstitial compartment", which decreased from 10/1 to 3.6/1.

To test for interference with the bioavailability of Steroid Acute Regulatory Protein (StAR), a major factor involved in the transport of cholesterol from the outer to the inner mitochondrial membrane (27), and of the steroidogenic enzymes, cytochrome P450 side-chain cleavage enzyme (P450scc) and cytochrome P450 17α-hydroxylase-17, 20-lyase (P450c17), their expression was assessed at the mRNA and protein levels (10). Interestingly, the expression at the mRNA level was

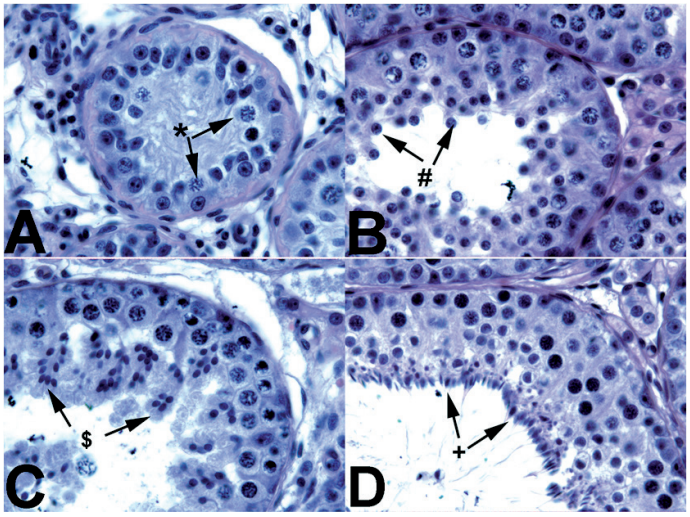

Fig. 4. A. Tubulus seminiferus contortus in a downregulated testis; arrest of spermatogenesis at the level of spermatogonia and primary spermatocytes (*); B. – D. testicular histology after the abolition of downregulation: B. arrest of spermatogenesis at the level of round spermatids (#), C. elongating spermatids ($), D. normal spermatogenesis with fully elongated spermatids (+) (according to (12))

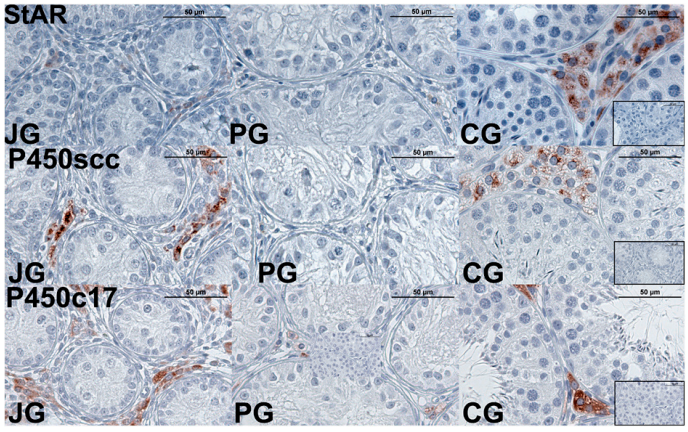

Fig. 5. Immunostaining for StAR, P450scc, and P450c17 in testes. JG, example of juvenile dogs; PG, example of fully downregulated dogs treated with a GnRH-agonist implant containing 6.3 mg buserelin acetate; CG, example of adult untreated controls; negative controls given as inserts (magnification, × 400) (according to (10))

lower, but not significantly different from that in the control group (Tab. 1). Concerning the expression at the protein level, as assessed by immunohistochemistry, data from the control testis clearly showed that immunopositive staining was restricted to Leydig cells. However, other than in the control testis, in the downregulated testis immunopositive staining was virtually absent for StAR and P450scc, and only slightly expressed for P450scc (Fig. 5, group PG). These observations indicate that downregulation affects the whole steroidogenic apparatus, but, as it seems, primarily with respect to post-translational processes.

The expression of the androgen receptor (AR) renders an organ susceptible to androgenic activity. As reviewed by Goericke-Pesch et al. (10), it is generally accepted that Sertoli, Leydig and peritubular cells express the AR. Some new light on the likely mechanisms controlling spermatogenesis is shed by the finding of Goericke-Pesch et al. (10) that also some spermatogonia seem to express the AR, as revealed by double-immunostaining for the AR and vimentin (Fig. 6), the latter clearly identifying Sertoli cells. Downregulation had no effect on the expression of the AR at the mRNA level. However, with respect to Sertoli cells, the classical target cells for androgenic activity, as revealed by immunohistochemistry, translation was apparently affected, since the number of AR-positive Sertoli cells was significantly decreased in the downregulated testis. The percentage of AR-positive Sertoli cells (median value) in the downregulated testis varied between 0 and around 17%, whereas about 100% of Sertoli cells were AR-positive in the control group. The number (median value) of AR-positive Sertoli cells with nuclei in basal position was around 95% in the control testis, and less than 1% in the downregulated testis. Thus, similar to the steroidogenic apparatus, downregulation with GnRH agonists seems to result primarily in the loss of or a significant reduction in AR expression at the protein level. The more or less unaffected expression at the mRNA level may be interpreted as the readiness of the system to upregulate quickly after the abolition of downregulation.

Tab. 1. Relative gene expression (ratio; x/SD) as determined for StAR, CYP11A1 (P450scc) and CYP17A1 (P45017c)

Parameter	Group		
	DGA	CG	JG
StAR	0.52/0.19*	1.07/0.38*	3.98/2.49**
CYP11A1	0.16/0.02*	1.14/0.59*	5.03/4.50*
CYP17A1	0.47/0.19*	1.14/0.61*	7.01/2.61**

Explanations: DGA: testes at downregulation, 5 months after the implantation of *Gonazon*®, a slow-release implant containing 18.5 mg of the GnRH agonist azagly nafarelin. CG – Control Group, JG – Juvenile Group (10)

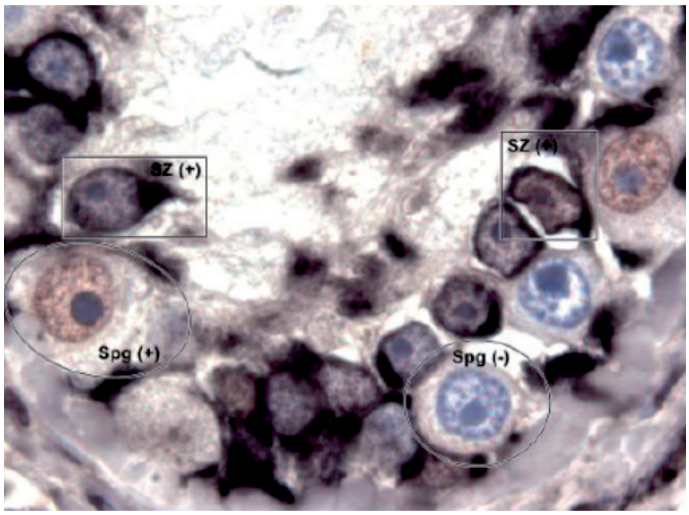

Fig. 6. Double-immunostaining for the AR and vimentin. A testis of a dog treated with a GnRH-agonist implant containing 18.5 mg azagly-nafarelin (magnification, × 1000, Spg(–), AR negative spermatogonia; Spg(+), AR positive spermatogonia; SC(+) vimentin positive sertoli cell)

The downregulated testis vs. the juvenile testis

The above observations clearly show that the downregulated testis differs from the juvenile testis. The two have been compared by Goericke-Pesch et al. (10). In the juvenile testis, apart from the well-established lack of spermatogonia and the presence of gonocytes, the steroidogenic enzymes P450scc and P450c17 are well expressed also at the protein level, unlike in the downregulated testis. However, similarly as in the downregulated testis, the expression of StAR – as indicated by immunohistochemistry – is virtually absent (Fig. 5, group JG). The expression of the AR at the mRNA level was clearly demonstrated with qPCR for the juvenile testis yielding a ratio amounting up to about 30% of what has been observed in the downregulated and control testes. However, immunostaining for the AR was negative. The lack of AR protein renders the juvenile testis insensitive to androgens, and the lack of StAR protein seems to be a key barrier with respect to the synthesis of steroid hormones. Both situations must be overcome during sexual maturation.

Recrudescence of testicular function

With the implant remaining *in situ*, the onset of testicular recrudescence may be observed after the loss of clinical efficacy, which may be stretched over time. The onset may be better defined after the removal of the slow-release GnRH-agonist implant. Relevant studies were performed with the use of *Gonazon*®, a slow-release implant containing 18.5 mg of the GnRH agonist azagly nafarelin (7, 12, 20). Testosterone concentrations (Fig. 7) increased rapidly, reaching pre-treatment values after 7 weeks, followed by a further increase until week 16. The availability of LH (Fig. 3, EPIV) reached pre-treatment values at the latest in week 12. This was paralleled by a testicular and prostatic regrowth. The onset became detectable about 6 weeks after implant removal, and pre-treatment values were reached between weeks 16 and 20 with a tendency for a further increase in the case of the prostate. Following implant removal, semen parameters were back to the physiological range after 29/30 weeks with a tendency for improvement in the parameters "% live spermatozoa", "% forward motility" and "% normal morphology" (11).

These observations clearly demonstrate that testicular endocrine function recovers rapidly after the abolition of downregulation. The course of testosterone concentrations points towards a rebound phenomenon, since the pre-treatment levels are exceeded. This continued testosterone increase must be seen as the reason for the prostatic growth exceeding pre-treatment values. Interestingly, semen quality was not merely restored, but actually improved in three parameters. Thus, the reorganisation of spermatogenesis, starting with spermatogonia, the main population of germ cells observed in the downregulated testis (see above), may be seen as a process which also includes some readjustments compared to the situation before treatment with the GnRH-agonist implant. Nevertheless, further observations are

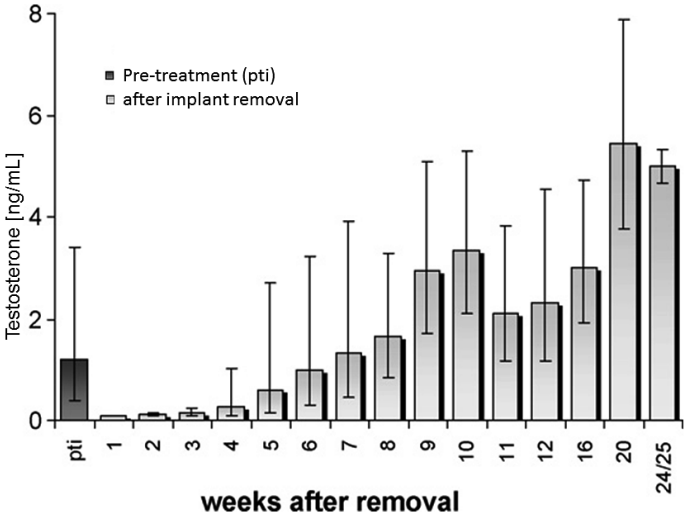

Fig. 7. Testosterone concentrations in peripheral plasma after the removal of a GnRH-agonist implant containing 18.5 mg azagly-nafarelin compared to pre-treatment levels (pti) (according to (20))

necessary to validate this hypothesis and the statement that spermatogenesis is fully regained. Ludwig el al. (20), as well, report that one dog – when semen quality tended to return to normal – developed an oligozoospermia, and that another dog remained azoospermic. This dog was castrated 40 weeks after implant removal, and a histological evaluation of the testis showed normal spermatogenesis. Thus the authors conclude that azoospermia was not a treatment-related effect, but rather an obstruction of the efferent ducts.

Apart from steroid hormone synthesis, there is still only little information available regarding the intratesticular events underlying testicular recrudescence. Up-regulation is clearly a rapid process. Following the abolition of downregulation by implant removal and the castration of dogs in 3-week intervals, Goericke-Pesch et al. (12) observed round spermatids after 3 weeks (Fig. 4B), whereas full spermatogenesis was re-established after 9 weeks (Fig. 4D). The formation of round spermatids 3 weeks after implant removal was paralleled by a full re-establishment of the steroidogenic capacity as indicated by the area of Leydig cell nuclei, which returned to control group levels (control group: 32.2 ± 1.8; at implant removal: 21.9 ± 2.3; three weeks after implant removal: 31.7 ± 3.1 μm^2) and the fully up-regulated expression of StAR, P450scc and P450c17 at both the mRNA level and the protein level (7). In the aforementioned study by Goericke-Pesch et al. (12), the availability of LH increased from 0.2 (2.15) at implant removal to 1.11 (1.7) ng/ml [x_g(DF)] three weeks later, and the corresponding values for testosterone were 0.1 (1.8) and 2.1 (2.3) ng/ml [x_g(DF)] with no further increase during the following 9 week observation period. Responsiveness to testosterone was clearly re-established as the number of AR-positive staining Sertoli cells was back to normal ($99.1 \pm 0.5\%$). These observations clearly show that the time window when the onset of testicular recrudescence may be observed is very narrow, and certainly less than three weeks. Once the "machinery" has started to work

at full speed, the process of spermatogenesis follows the normal cycle, lasting for about 61 days (6).

Concluding remarks

By now, the downregulation of testicular function in the dog with the use of slow-release GnRH-agonist implants is a well-defined process from the clinical point of view and with respect to the underlying mechanisms of action. Downregulation may therefore be defined a safe approach for a reversible, temporary hormonal castration. An appropriate veterinary drug is available on the market, but, as with any new drug becoming available in veterinary or human medicine, attention must be given to possible side effects.

The status of the downregulated testis has been well defined with respect to the impairment of steroido- and spermatogenesis and the responsiveness to androgens. It is evident that the downregulated testis does not resemble the juvenile testis, but rather the situation observed in the testis of seasonal animals out of season, such as the roe deer or Djungarian hamster, where LH, FSH, and testosterone are basal out of season (1, 23, 24, 26) with spermatogenesis arrested at the level of spermatogonia and the area of the Leydig cell nuclei being reduced (2, 3, 14, 25). Also the observed movement of Sertoli cell nuclei from a more basal to a more luminal position has been described for seasonal breeding animals (25).

As the seasonal up-regulation of testicular function is a process occurring regularly in yearly intervals, generally resulting in full fertility, it can be expected that the same applies to the downregulated testis, and the few observations made so far after repetitive treatments seem to confirm this conclusion (22). As indicated above, a tendency for a somewhat improved semen quality was observed after the abolition of downregulation and the recrudescence of testicular function. Thus the downregulated canine testis and testicular recrudescence might serve as a good model for gaining further information on the intratesticular processes controlling spermatogenesis. The studies carried out so far have clearly shown that the time window to assess endocrine control mechanisms is very narrow, and that endocrine up-regulation occurs within three weeks. However, this does not account for spermatogenesis, which is fully re-established only after about 9 weeks. There are still more questions than answers – for example, how does downregulation affect the blood-testis barrier? Further studies should account for the observations made so far.

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