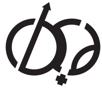

Angiogenic and steroidogenic responses of the corpus luteum after superovulatory and stimulatory treatments using eCG and FSH

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Abstract

The corpus luteum (CL) is a pivotal organ for the success of reproductive function and a main target of assisted reproduction techniques. Moreover, increases in progesterone production and CL volume have been repeatedly reported after the use of exogenous gonadotropins administered to improve the quality of the dominant follicle (stimulatory treatment) or to increase the number of ovulated follicles (superovulatory treatment). This review aims to discuss the CL-related molecular, cellular and tissue mechanisms associated with the improvement of reproductive performance achieved under superovulatory and stimulatory treatments in cattle. Here, we report that FSH (follicle stimulating hormone) but not eCG (equine chorionic gonadotropin) induce vascularization at the molecular level despite the increase in CL volume in response to both gonadotropins. Increases in plasma progesterone concentrations per CL in response to a stimulatory but not superovulatory treatment with eCG as well as cellular and molecular alterations that support luteal function are also reviewed. Whereas practitioners make use of hormonal protocols aiming to increase pregnancy rates, we focus our efforts on uncovering the cellular and molecular mechanisms that are triggered by such successful strategies, hoping our findings will contribute to the achievement of the next successful step.

Keywords: bovine, corpus luteum, eCG, FSH, vascularization.

Introduction

The corpus luteum (CL) is an endocrine gland capable of producing progesterone (P4) and support gestation from the beginning until the end of the second trimester in ruminants or until the very end in humans and dogs. In ruminants, when gestation begins, P4 binding to its receptor in the uterine epithelium and in the embryo triggers two different cascades: the first one enhancing the ability of uterine glands to secrete substances into the uterine lumen, that are collectively known as histotroph, to provide nourishment of the embryo, and the second one, in the embryo itself, enabling it to increase interferon-tau (INF-tau) production and consequently inhibit PGF2α uterine secretion (Soloff *et al*., 2011). Although for humans and dogs the histotroph cascade is also started by P4, there is no production of IFN-tau nor a need to prevent PGF2α production in the beginning of gestation by either species (Galabova-Kovacs *et al*., 2004; Soloff *et al*., 2011). In these species, although P4 is known to act in an endocrine fashion on the uterus, it is recognized mainly by acting in an autocrine and paracrine manner on the CL, where it is considered an important luteotropic factor (Maybin and Duncan, 2004; Papa and Hoffmann, 2011).

It is believed that the above-mentioned processes are disrupted when CL does not produce enough P4 and gestation is interrupted. Gestational failures are responsible for important economic losses on the dairy and beef industries. In order to minimize losses, hormonal protocols to manipulate the estrous cycle have been developed (Carvalho *et al*., 2008; Baruselli *et al*., 2011) aiming to increase pregnancy rates. Timed artificial insemination (TAI) programs have efficiently overcome the issue of estrus detection inaccuracy, which corresponds to a major limitation in the cattle industry. Since estrus detection is not necessary in TAI programs, service rates reach 100%, as every animal is inseminated within a fixed interval after induction of ovulation. In addition to the synchronization factor, TAI protocols in association with gonadotropin treatment have resulted in increased conception rates probably due to increased plasma P4 concentrations during early diestrus (Binelli *et al*., 2001; Bó *et al*., 2002; Sá Filho *et al*., 2010; Sales *et al*., 2011). Protocols using decreasing doses of FSH (follicle stimulating hormone) after TAI to promote superovulation improved embryos collected/flush by 46% (Carvalho *et al*., 2002). More recently, Baruselli and collaborators (Martins *et al*., 2006; Baruselli *et al*., 2008) replaced the use of FSH to induce superovulation by eCG (2000 IU). The main advantage of using eCG is that only a single administration is needed, on day 4 after the beginning of the TAI protocol. Additionally, the same group started using eCG to stimulate growth of the preovulatory follicle (400 IU). Ovulation of a larger preovulatory follicle is usually associated with increases in the CL volume and progesterone concentration (Binelli *et al*., 2001; Sá Filho *et al*., 2010; Fields *et al*., 2012). Success of the aforementioned treatments was reported

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by several other groups (Bó *et al*., 2002, 2011; Souza *et al*., 2009; Sales *et al*., 2011) and the same intriguing question remained: What are the mechanisms by which plasma P4 and CL volume increase in response to eCG?

This review contains results from two major studies conducted in collaboration with Prof. Baruselli's group that accessed the CL morphophysiology under FSH and eCG treatments. The first study assessed microvascular density, capillary arrangement and expression of vascular related growth factors in water buffalo CL after a superovulatory treatment. In the second study, we established the aim of uncovering tissue, cellular and/or molecular mechanisms responsible for the increase in plasma P4 concentrations and CL volume in response to eCG treatment, and associated increase in pregnancy rates. To accomplish the overall aim we established as specific aims to measure CL volume, mitochondrial density, number and size of different luteal cell populations and the global gene expression of corpora lutea (CLs) collected on day 6 postovulation from superovulated, stimulated and untreated control cows.

CL morphofunctional features under FSH superovulatory treatment

Water buffalo CLs were analyzed after treatment using 200 mg of FSH administered from day 0 (start of treatment with FSH) to day 3 as equal doses twice daily in decreasing amounts. Details of the animal handling protocol can be obtained in Carvalho *et al*. (2002) and Papa *et al*. (2007). After studying the microvascularization by morphometry and corrosion casts we observed a 3-fold increase of microvascular density in superovulated CLs as well as an increase of arterial and venous capillary beds in these CLs (Moura, 2003). These observations, which corroborated those of other groups describing increased vascular-related growth factor expression after gonadotropin treatment (Christenson and Stouffer, 1997; Laitinen *et al*., 1997; Wulff *et al*., 2000; Sasson *et al*., 2003) lead us to verify the expression of the VEGFA and the FGF2 systems in the superovulated CL model in buffaloes. At the protein level, immunohistochemistry (Prado, 2004; Papa *et al*., 2007) and western blot (Fatima, 2008) analyzes revealed a marked increase in the expression of the two most important vascular growth factors (Schams and Berisha, 2004) in the superovulated CLs compared to untreated control animals. Interestingly and unexpectedly (Shin *et al*., 2005; Herr *et al*., 2010), mRNA expression of the great majority of the other growth factors and their receptors (VEGF, FLT1, KDR, FGF2, FGFR1 and FGFR3) was decreased in the superovulated CLs (Papa *et al*., 2007; Fatima, 2008).

Trying to reproduce the superovulatory treatment using FSH *in vitro*, Fatima *et al*. (2011) cultured bovine and buffalo granulosa lutein cells in *vitro* and observed a similar protein expression increase after immunocytochemistry to detect VEGFA and VEGF receptor 2 (VEGFR2). There was a decrease in the mRNA abundance of VEGFA but not for VEGFR2. Although an *in vitro* model is not always suitable to reproduce *in vivo*/*ex vivo* changes (Broussard *et al*., 1995), it is clear that FSH superovulatory treatment does increase protein expression of vascular-related growth factors in CL, suggesting that this is one potential mechanism involved, directly (Yamashita *et al*., 2008) or indirectly (Dickson *et al*., 2001; Kashida *et al*., 2001), in the regulation of P4 production in response to gonadotropins. In fact, increased plasma P4 concentrations per CL was also reported for this model (Papa *et al*., 2007) and we described an increase in luteal cell organelles (Artoni *et al*., 2004) involved in P4 synthesis in superovulated CLs. Under investigation by our group at the moment are different cellular pathways responsible to translate mRNA into protein that could be affected by the treatment with FSH.

CL morphofunctional features under eCG stimulatory and superovulatory treatments

To study the effects of eCG on luteal cells and tissue after a stimulatory or superovulatory treatment, the following experimental design was used. Cows (*Bos indicus*) were distributed randomly in three groups (control, n = 5; stimulated, n = 6; and superovulated, n = 7) and submitted to estrous cycle synchronization using progesterone devices and estradiol benzoate (considered day 0 of the protocol). The stimulated group received 400 IU of eCG on day 8, whereas the superovulated group received 2000 IU of eCG on day 4 after the beginning of the TAI protocol. Animals were slaughtered on day 6 after ovulation and CLs were collected. The CL volume was measured postmortem using a caliper and the formula for an ellipsoid [3/4pi (width/2) (height/2) (depth/2)]. CL volume for superovulated animals was calculated using mean values of 3 CLs per animal. Plasma P4 concentrations were assessed by RIA and calculated on a CL basis (P4 divided by CLs number). Plasma P4 per CL was higher in the stimulated group, while CL volume reached its largest values in the superovulated group (Fatima *et al*., 2010).

Next, we quantified the abundance of vascular-related growth factors. Protein expression of those factors was expected to increase in response to eCG treatment, according to data described above for CLs from FSH-treated animals. Corpus luteum expression of VEGF, FLT1, KDR, FGF2, FGFR1 and FGFR2 was assessed at the mRNA level by TaqMan quantitative real-time RT-PCR (qPCR). Primers and probe sets used were obtained from Life Technologies (Carlsband, CA, USA) and the specifications were published elsewhere (Campos *et al*., 2010). Quantitative PCR was performed as described by Campos *et al*. (2010) and alpha-tubulin was chosen as the reference gene. Relative expression was calculated (Pfaffl, 2001) after LinReg PCR data

processing (Ramakers *et al*., 2003). From the six studied target genes, only FGFR2 was increased in the stimulated group in relation to control (P < 0.05). According to Castilho *et al*. (2008) and Guerra *et al*. (2008) different isoforms of FGFR2 are expressed in a time-dependent manner in bovine CL throughout the estrous cycle, mediating biological responses such as stimulation of luteal cell proliferation, steroidogenesis and angiogenesis, in response to FGF2 binding (Gospodarowicz *et al*., 1977; Miyamoto *et al*., 1992; Robinson *et al*., 2008). Castilho and colleagues also suggested that FGFR2B is involved in the mechanism of structural luteolysis of bovine CL (Fatima *et al*., 2010). Further studies would be necessary to clarify whether the observed increase in FGFR2B gene expression could be related to the observed phenotype of stimulated animals.

The VEGFA system was also analyzed for protein localization and content by immunohistochemistry and Western blot, respectively. The immunohistochemistry procedure used in our studies was as described previously by Papa *et al*. (2007) including antibodies specifications and dilutions. The same antibodies were used to Western blot analysis (for details, please see Fatima, 2008). The VEGF, FLT1 and KDR protein expression assessed by Western blot did not change significantly among groups (data not shown). However, when the immunolocalization was analyzed we observed a stronger staining for VEGF in small luteal cells of superovulated compared to control and stimulated animals (Fig. 1). Although the expression of VEGF and its receptors has been studied extensively in bovine corpus luteum (Berisha *et al*., 2000; Wulff et al., 2000; Endo *et al*., 2001), this is the first report assessing their regulation after eCG treatment. Based on our buffaloes data, we expected to find both VEGFA and FGF2 systems to be altered, not only in superovulated cows, but also in stimulated ones, since gonadotropins increased VEGF expression. Nevertheless this difference could be species-specific (buffalo *vs.* cattle). Moreover, it has been demonstrated that VEGF and FGF2 increase the secretion of progesterone from luteal cells in several species (Gospodarowicz *et al*., 1977; Berisha *et al*., 2000; Yamashita *et al*., 2008), indicating that both factors are involved in formation of CL and control of P4 secretion. Work carried out using anti-FGF2 or anti-VEGF antibodies reported a 50% decrease in the volume of the bovine CL and P4 production (Yamashita *et al*., 2008).

In light of these results, we decided to assess the global gene expression of the luteal tissue from our experimental groups by microarray analysis to identify potential relationships between the reported alterations in P4 plasma concentrations and CL volume and the transcriptional profiles observed. Global gene expression of the CL collected from control, stimulated and superovulated cows was determined using the Affymetrix GeneChip Bovine Genome Array. Overall, more than 300 transcripts were differentially expressed between the control and stimulated animals and more than 200 between control and superovulated animals (1.5 fold change, P < 0.05). Quantitative PCR and Western blot were also carried out to validate microarray expression data for some genes. Among transcripts differentially expressed, there were changes in genes involved in lipid and carbohydrate biosynthesis, cellular signaling, immune response, post-translational modification, tissue development, cell morphology, growth and proliferation. After analysis of the main cellular pathways altered by the eCG treatments, the peroxisome proliferator-activated receptors (PPARs) pathway was significantly represented by the differentially expressed genes, including PPARG, which was increased in eCG treated cows. The PPARs are transcription factors that play a central role in the regulation of lipid metabolism and may also participate in regulation of development and regression of the CL (Komar, 2005). The main role of PPARG is to arrest the cell cycle of luteal cells to maintain their differentiated state (Viergutz *et al*., 2000). The integrated control of this pathway is probably involved in the regulation of energy homeostasis and metabolism to promote CL growth and endocrine function. Furthermore, the differential expression of PPARG could also be related to an increase in substrate availability (cholesterol) for P4 synthesis. Thus, the increased P4 concentration in eCG-treated cows may be a result of the combination of increased CL volume, regulation of lipid metabolism and the alteration of the cell machinery involved in luteal P4 synthesis. To corroborate with this hypothesis, the steroidogenic acute regulatory protein (STAR) gene and protein expression were also increased in the CL of eCG treated cows (Fatima *et al*., 2011). STAR carries cholesterol from the outer to inner mitochondrial membrane, which is the rate-limiting step in the synthesis of progesterone (Stocco and Clark, 1996; Stocco *et al*., 2005).

To reinforce microarray data, a morphological study was carried out (Fatima *et al*., 2010) to measure the number and volume of small and large luteal cells, mitochondrial density and volume as well as density of spherical and elongated mitochondria in control, stimulated and superovulated CLs. After fixation in 2.5% glutaraldehyde or in 4% paraformaldehyde, CLs were processed for transmission electron and light microscopy, respectively. In superovulated cows, the assessment of mitochondria by stereology showed increased mitochondrial volume per CL, and morphometrical analysis showed increased number of SLCs and diameter of LLCs. In the stimulated group, density of spherical mitochondria was increased compared to the other two groups, as well as the number of LLCs. Our morphological data corroborates with the observed capacity of eCG in increasing plasma P4 concentrations under stimulatory treatment and CL volume under superovulatory treatment. An increase in

spherical mitochondria density together with the number of LLCs in stimulated cows accounted to higher plasma P4 concentrations (Fields and Fields, 1996). LLCs are the main P4-producing cells in the CL (Fields and Fields, 1996; Niswender *et al*., 2000) and luteal cell features related to steroidogenesis, such as an increased mitochondrial capacity for pregnenolone production after eCG treatment, have already been described (Tuckey and Atkinson, 1989). In our study, the stimulatory treatment induced morphological changes that enabled the CL to produce more P4 in relation to total CL volume. On the other hand, the superovulatory treatment induced changes related to volume increase, as increased number of SLCs and total volume of mitochondria.

Concluding remarks

Hormonal protocols are broadly used to optimize reproductive efficiency. FSH seems to be more efficient as a superovulatory stimulus, since vascularization, a fundamental step for organs in the process of growing and becoming metabolically active, and progesterone increase were stimulated by this gonadotropin. Nevertheless, these features are not necessarily useful for donor cows, which will not bear embryonic/fetal development. On the other hand, eCG can be used in superovulation protocols in a single administration, as well as in the stimulation of the dominant follicle, aiming to increase P4 production by the resulting CL. The superovulatory response to eCG treatment is variable among cows, although the number of ovulated follicles increases; eCG prepares the ovary and follicle to support this increase. The stimulatory response to eCG treatment (i.e. P4 production) is more homogeneous and furthermore improves pregnancy rates (Fig. 2). The positive impact of eCG on pregnancy outcome is likely due to eCG ability to induce changes at the molecular, cellular and tissue level that are related to increased functional capacity of P4 synthesis.

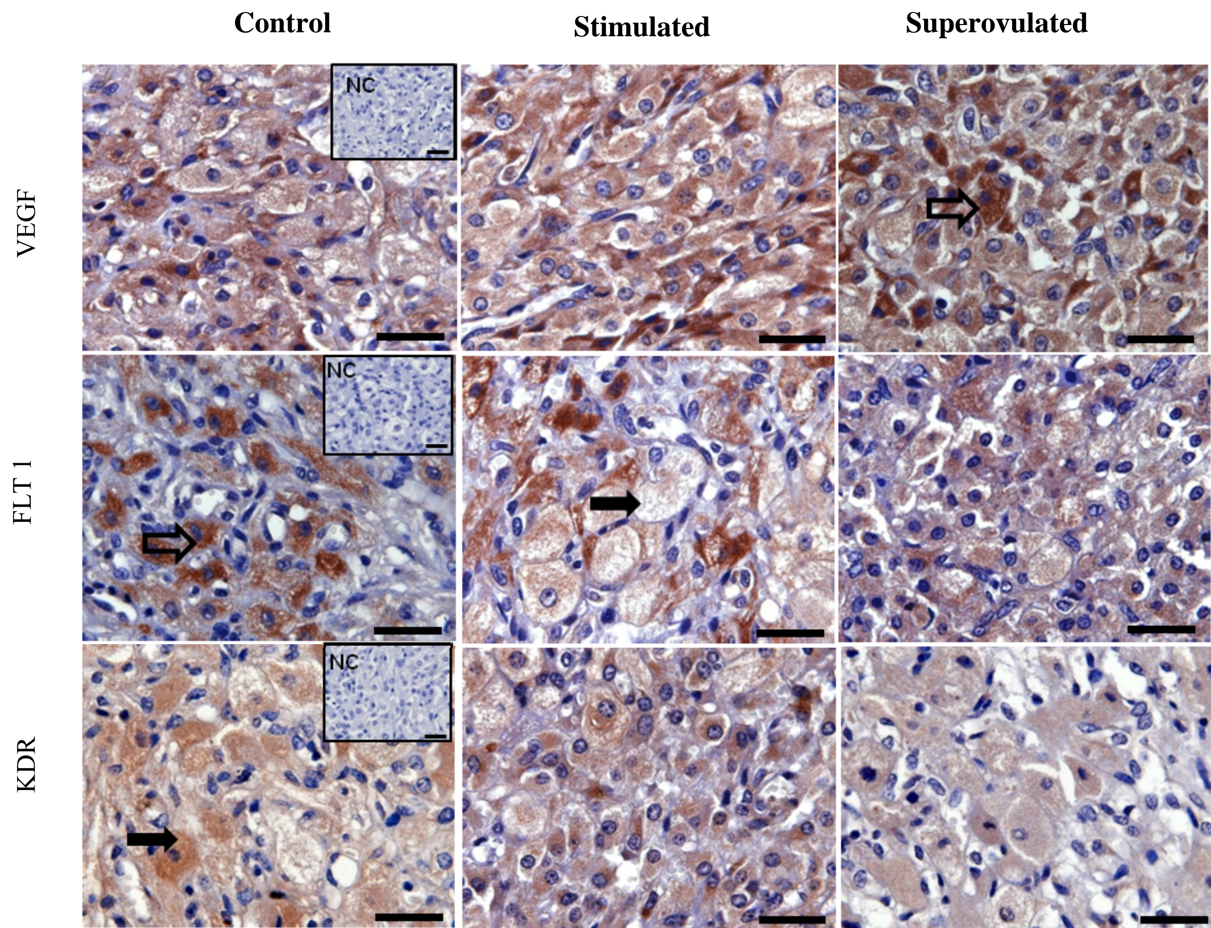

Figure 1. VEGF, FLT1 and KDR expression in bovine CL from day 6 after ovulation, detected by immunohistochemistry. Positive signals can be observed as the orange/brown color in the cytoplasm of the large (closed arrow) and small (open arrow) luteal cells in the control, stimulated and superovulated animals; (NC) Negative control. Bars = 50 µm.

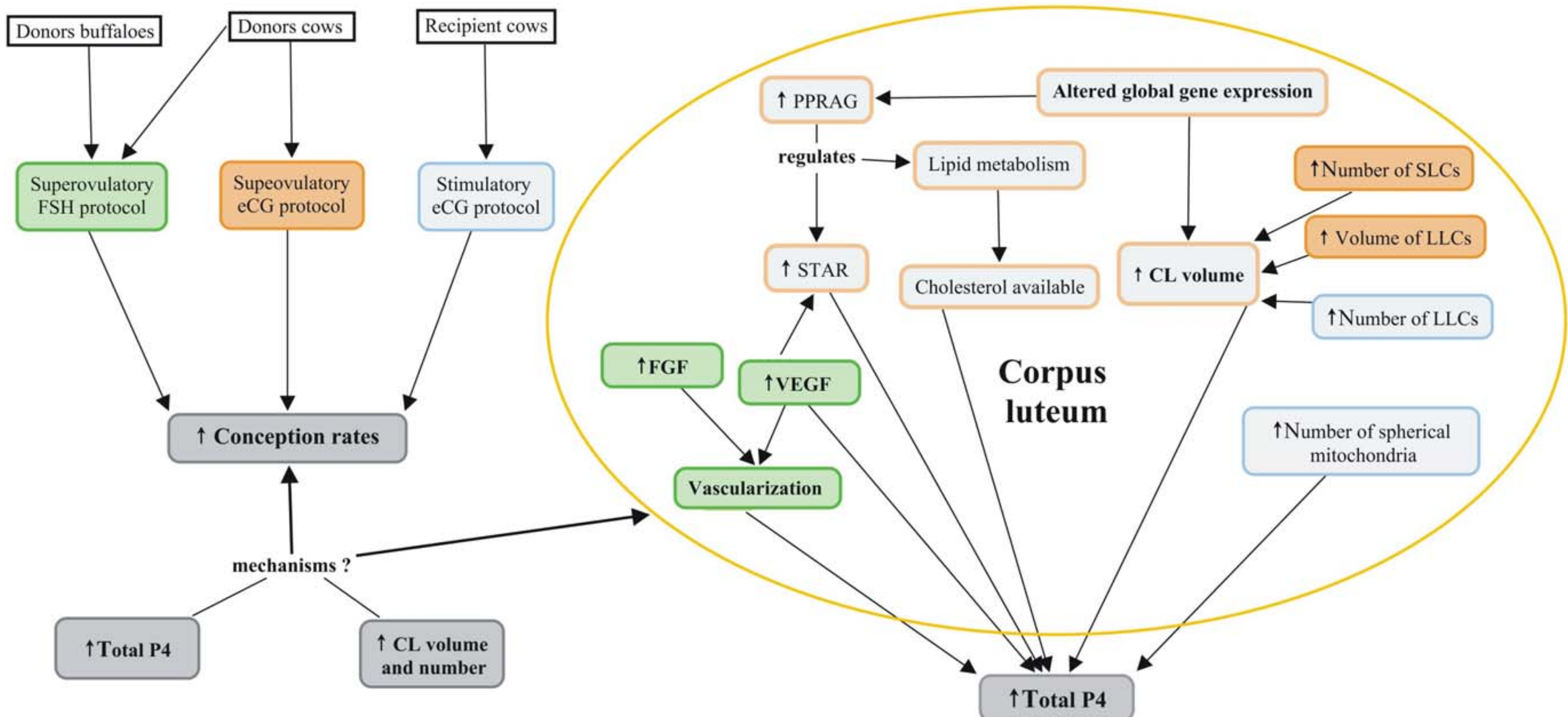

Figure 2. Simplified diagram showing the proposed sequence of events involved in the increase of CL volume and plasma P4 observed after treatments with eCG and FSH. In the left the events related to different treatments: stimulatory (blue box) and superovulatory (orange box) using eCG in cows and superovulatory (green box) using FSH in water buffaloes. The events occurring in the CL under eCG and FSH treatments are explained in the right (yellow circle). The color of the boxes inside the CL are related to the treatment used since not all events observed in the CL occurred under all treatments. SLCs: small luteal cells; LLCs: large luteal cells; P4: progesterone; ↑: enhance or promote.

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