# LHRH inactivation by reconstituted horse and fetal bovine sera: assessment by reduction of immunoreactivity and biological activity in pituitary cell cultures

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Lyophilized horse and fetal bovine sera are commonly incorporated into the growth media used for primary pituitary cell cultures. LHRH degrading activity has been assumed to exist in these preparations but has not actually been demonstrated. During our studies with pituitary cultures, it became necessary to ascertain if LHRH inactivating activity could be demonstrated in these sera. Luteinizing hormone releasing hormone (LHRH) was preincubated in either serum-free medium or medium containing fetal bovine and horse serum. Whether LHRH was lost during these incubations was assessed by diminished immunoreactivity as indicated by radioimmunoassay (RIA) and by diminished biological activity as indicated by reduced release of LH from pituitary cell cultures. Both the RIA and bioassay results indicated LHRH inactivating activity; the loss of LHRH could be prevented by inclusion of bacitracin in the incubations.

*LHRH* *Pituitary culture* *LH* *Bovine serum* *Horse serum* *LHRH inactivation*

## 1. INTRODUCTION

We have reported a portion of our work studying luteinizing hormone releasing hormone (LHRH) effects in the release of gonadotropins from monolayer pituitary cell cultures [1,2] and from superfused pituitary cultures attached to Cytodex I beads [3–5]. Prior to LHRH exposure in these and similar studies, the serum-containing growth medium was replaced by a highly defined medium. Because the serum seems to serve a vital but undefined role in culture survival, we were led to ponder how cellular response to a given LHRH regimen would compare in the defined serum-free medium as opposed to a complete serum-containing medium such as that in which the cells were initially grown; however, whole serum from humans and rats has been reported to contain LHRH inactivating activity [6]; it has not been reported whether similar activity exists in the lyophilized commercially available horse and fetal bovine sera commonly incorporated into growth media. Here we investigate LHRH inactivation in growth medium containing lyophilized horse and fetal bovine serum.

## 2. MATERIALS AND METHODS

### 2.1. *Studies in fetal bovine and horse serum to detect the presence of LHRH cross-reacting material*

Initial studies were conducted to determine if horse and fetal bovine sera contained material which depressed binding in the LHRH radioimmunoassay (RIA). Following reconstitution of the lyophilized products, each serum was assayed individually prior to charcoal stripping; in addition, the sera combined in equal proportion (the proportions in which they were included in the growth medium) were assayed prior to as well as subsequent to charcoal stripping.

### 2.2. *Preparation of complete growth medium*

The culture medium was composed of Dulbecco's Modified Eagle Medium (DMEM) (Gibco no.320-1965) supplemented with 15% charcoal-

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stripped fetal calf serum (Difco no.5065-67) and 15% horse serum (Difco no.5357-67). The sera were lyophilized when purchased and subsequently reconstituted in distilled water (Gibco no.670-5230), Penicillin–streptomycin (Gibco no.600-5140), fungizone (Gibco no.600-5295) and non-essential amino acids (Gibco no.320-1140) were added at 1% concentration.

### 2.3. *Preincubation of LHRH with serum-containing growth medium*

In order to assess the effect of horse and fetal bovine serum on the stability of LHRH, 50-ng quantities of LHRH were incubated at 37°C in either 1 ml 0.01 M phosphate-buffered saline + 0.1% gelatin (PBS + gel) for 60 min or 1 ml complete growth medium for 15, 30 and 60 min. 60-min incubations in complete growth medium containing 1.0 mM bacitracin were also conducted in order to ascertain that, if LHRH loss were observed, it was indeed due to peptidase enzyme activity. At the end of the incubations, the reactions were stopped by heating at 100°C for 3 min. After centrifugation to remove precipitated protein, aliquots of the medium were assayed by RIA and bioassay techniques for indications of diminished LHRH. Each serum was not assayed individually for LHRH inactivating activity because they were not incorporated into growth medium individually.

### 2.4. *Radioimmunoassay procedures for LH and LHRH*

Luteinizing hormone (LH) levels were estimated in a double antibody RIA utilizing NIAMDD reagents as previously described [2,9]. The intra-assay coefficient of variation did not exceed 10%, while the inter-assay coefficient of variation did not exceed 15% ($n = 18$). LHRH was iodinated according to the lactoperoxidase–$H_2O_2$ method [10,11]. LHRH levels were estimated in a double antibody RIA as previously described [12]. The intra-assay coefficient of variation did not exceed 10%, while the inter-assay variation did not exceed 15%.

### 2.5. *Bioassay to detect LHRH inactivation*

Monolayer pituitary cell cultures [2] were used to conduct the bioassay for LHRH inactivation. These studies utilized intact, Holtzman (Madison, WI) albino male rats at 60 days of age. They were maintained under day cycles of 14 h light and 10 h darkness (lights on at 06.00 and off at 20.00 h). The rats and the cell cultures were prepared as previously described [2,5]. At 3 days (72 h), the complete serum-containing growth medium was substituted by DMEM (either 0.9 or 0.95 ml) without serum; the source of LHRH, either control preincubations (in PBS + gel or in heat-inactivated growth medium) or experimental preincubations (unheated growth medium) was then added, either in 100 $\mu$l or in 50 $\mu$l to make a total volume of 1 ml. Following a 3-h incubation, the media were removed and LH release was estimated by RIA. Duncan's New Multirange test [13] was used to judge the significance of decreased LHRH levels or decreased LHRH biological activity.

## 3. RESULTS AND DISCUSSION

Immunoreactive LHRH cross-reacting material was observed in serum (fig.1); prior to charcoal stripping (bar 1), such material was observed in horse serum while in bovine serum (bar 2) essen-

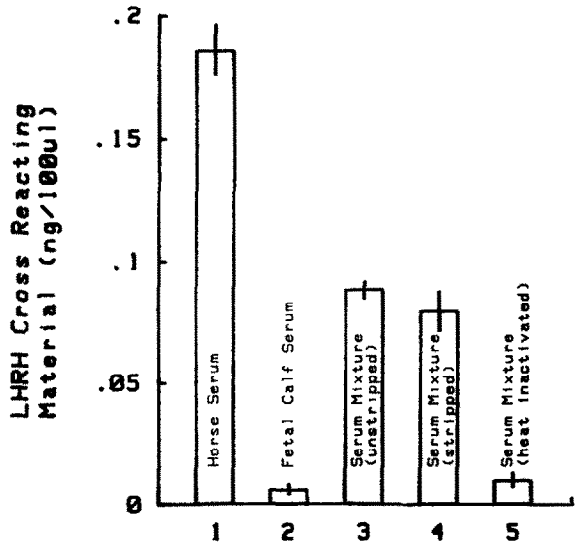


Fig.1. Immunoreactive LHRH cross-reacting material observed before charcoal stripping in (1) horse serum, (2) fetal bovine serum and (3) the two sera mixed in equal proportions, as well as (4) after charcoal stripping in the two sera mixed unheated, and (5) subsequent to 100°C for 3 min. In this and subsequent figures, values are plotted as the mean (± SE) of triplicate determinations.

tially no such material was found. Bars 3 and 4 indicated cross-reacting material in a mixture of equal parts of the two sera before and after charcoal stripping, respectively, and that mixing equal parts of the two sera diluted the substance in half. Immunoreactivity of the substance was eliminated by 3 min at 100°C (bar 5).

When LHRH at a concentration of 5 ng/100 $\mu$l was incubated in the presence of PBS + gel or heat-inactivated (100°C for 3 min) growth medium (fig.2; bars 1,2), RIA indicated no significant LHRH loss. When incubation was for increasing periods of time in the presence of complete growth medium (bars 3–5), progressive LHRH loss was observed; all 3 time points were significantly ($P <$ 0.01) lower than starting levels. If 1.0 mM bacitracin were included in the incubations (bar 6), the significant loss of LHRH was not detectable.

Monolayer cultures were used to determine biological activity in response to LHRH preincubated at a concentration of 5 ng/100 $\mu$l in the presence of buffer or complete growth medium (fig.3). The response to control (no LHRH) incubations (bars 1,2) indicated that growth medium containing stripped sera had no more effect on

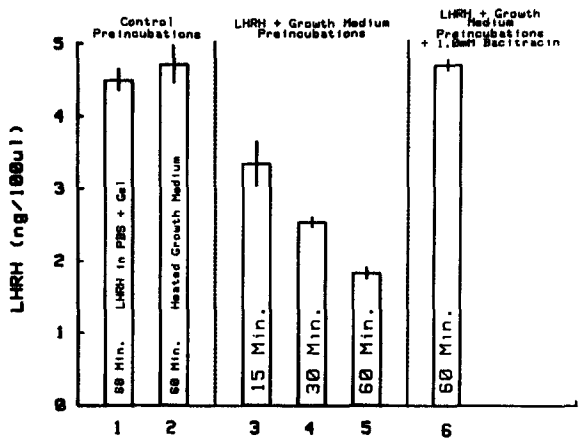


Fig.2. Effects on the RIA detectable levels of LHRH (original concentration = 5 ng/100 $\mu$l) subsequent to incubation in (1) PBS + gel, (2) heat-killed (100°C for 3 min) complete growth medium, as well as (3–5) effects following incubation for increasing periods of time in the presence of unheated complete growth medium in the absence and (6) in the presence, of 1.0 mM bacitracin. The length of incubation prior to heat inactivation (100°C for 3 min) is indicated within each bar. All incubations were at 37°C.

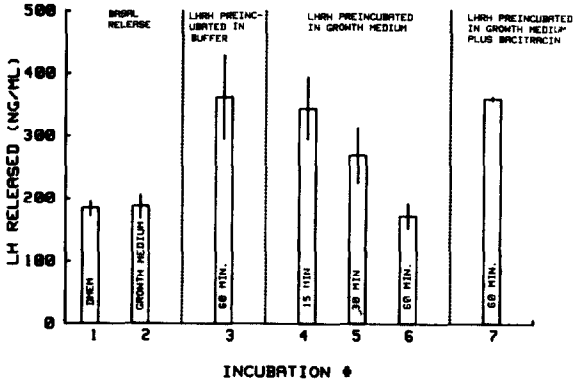


Fig.3. LH released from monolayer pituitary cell cultures derived from 60-day intact male rats in response to (1) DMEM only, (2) growth medium only, (3) 100 $\mu$l of an LHRH solution subsequent to 60 min preincubation in 0.01 M PBS + 0.1% gelatin (5 ng LHRH/100 $\mu$l), (4–6) 50 $\mu$l of an LHRH solution (5 ng/100 $\mu$l) diluted in growth medium and assayed for biological activity subsequent to 15, 30 and 60 min preincubation, respectively, in the absence and (7) in the presence, of 1.0 mM bacitracin. All preincubations were performed at 37°C.

basal LH release than did DMEM alone; the LHRH cross-reacting material (fig.1) therefore apparently had no biological activity as far as LH release was concerned. Bacitracin at 1.0 mM concentration in either DMEM or growth medium had no further effect on basal release (not shown). Bar 3 indicates the response to the LHRH buffer control dilution (5 ng LHRH). The LH release induced by 50 $\mu$l of LHRH solution (which would correspond to 2.5 ng LHRH in the absence of inactivating activity) preincubated in growth medium for 15, 30 and 60 min is shown in bars 4–6; the bioassay system reflected the previously discussed RIA indicated loss of LHRH. Inclusion of 1.0 mM bacitracin would prevent this loss of biological activity (bar 10).

In summary, horse serum was observed to contain material (2 ng/ml) which depressed binding in the LHRH RIA; this material was not detectable in fetal bovine serum. The material was heat labile, was not removed during charcoal stripping and had no LH-releasing biological activity. The presence of cross-reacting material has been reported by several investigators in different tissues [14–17]. Here, its identity was not investigated further; however, in view of the fact

that subsequent studies showed a loss of LHRH, it is conceivable that the cross-reacting material might actually be due to enzymatic tracer damage during the RIA incubation process. Subsequent to 15-, 30- and 60-min incubations at 37°C, initial concentrations of 5 ng LHRH/100 $\mu$l in growth medium indicated progressive losses of immunoreactive LHRH; comparable losses of biological activity were also noted as indicated by LH release. That the LHRH loss was due to peptidase activity was indicated by the fact that the inclusion of bacitracin (1.0 mM) in the incubations prevented significant loss of LHRH immunological or biological activity. The observed inactivation is especially of interest since it has been recommended in the literature [18] that fetal bovine serum be added to the incubation media from pituitary cell cultures in order to stabilize released LH. If one were interested in determining LHRH levels remaining subsequent to gonadotropin release, inaccurate values might be obtained. Some studies [19] indicate that fetal bovine serum exhibits no effect on LHRH-induced LH release from pituitary cultures; in view of the fact that the serum was removed prior to LHRH administration, caution should be exercised in attempting to apply these conclusions to a system in which serum and LHRH were utilized simultaneously.

To our knowledge, this is the first detailed report actually confirming LHRH inactivating activity in the lyophilized horse and fetal bovine sera which are widely incorporated into pituitary cell culture media; these studies have also indicated that this inactivating activity, as previously reported in other mammalian systems [18,20], is inhibitable by bacitracin. This may suggest methodological modifications in certain pituitary culture procedures.

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